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THE PROMOTION OF ABSCISSION BY AUXIN[1,2]

B. K. GAUR AND A. C. LEOPOLD

HORTICULTURE DEPARTMENT, PURDUE UNIVERSITY, LAFAYETTE, INDIANA

Though some scientific studies on the abscission of plant organs had started as early as 1860 (10), the role of the growth hormone in this process was first suggested by the work of Laibach in 1933 (6). He observed that orchid pollinia inserted in the split ends of petioles of debladed Coleus delayed abscission 11 to 20 days beyond that of untreated petioles. Soon after, La Rue (7) found that indoleacetic acid, pollen extracts or urine delayed petiole fall for a few days when they were applied to debladed petioles. He suggested that abscission resulted when the development of the abscission layer was not inhibited by growth substances produced in the leaf blades. Since then many practical uses have been made of the delaying action of auxin on abscission.

Myers (11, 12, 13) and Gardner and Cooper (4) by their deblading experiments in Coleus have essentially proved that auxin is responsible for the prevention of leaf drop. They concluded that the leaf blade was the source of the phytohormone naturally serving this function.

A new contribution was made when Shoji et al (15) proposed the auxin gradient theory of abscission. They made auxin determinations in bean leaves at various intervals preceding abscission, and found that although the auxin concentration in the blade started falling just before abscission, the concentration in the petiole itself remained unchanged. From this they deduced that a critical auxin concentration gradient across the abscission zone may be necessary to prevent the leaf from abscissing rather than the auxin concentration itself. Addicott and Lynch (1) further observed that whereas distal applications of auxin to debladed petioles prevented abscission, proximal ones induced it. This finding corroborated the gradient theory.

Auxin is generally considered to be an abscission inhibiting material, although no convincing quantitative studies to this effect have been carried out. In view of the fact that most growth functions, inhibited by higher concentrations, are also promoted by lower ones, it would seem worthwhile to investigate the quantitative aspects of auxin effects on abscission.

MATERIAL AND METHODS

Coleus blumei Benth var. Purdue Fern Marie and *Phaseolus vulgaris* L. var. Red Kidney were used as experimental plant material.

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[2] Journal paper No. 871, Agricultural Experiment Station, Lafayette, Indiana.

Coleus plants with eight pairs of expanded leaves were used. Each plant was debladed at the 3rd, 4th and 5th leaf pairs (counting from the youngest expanded leaf), the petioles being trimmed down to a uniform length of 2 cm. A drop of lanolin paste containing a specified concentration of auxin was applied to cover the cut surface of each severed petiole. Control plants were similarly prepared but treated with pure lanolin only. In the experiments with Coleus no special control of the greenhouse temperature was made, but all comparable treatments were made simultaneously, and so were subjected to the same fluctuations of temperature, light and humidity. Counts of petioles dropping from plants after treatment were made daily. In making these counts, the pots were tapped gently to dislodge any petiole which might be ready to abscise.

The first trifoliate leaves of Red Kidney beans obtained from 23- to 26-day-old plants were used. The terminal leaflet was not used, and was cut off at the point of its contact with the main petiole. The laminae of the two lateral leaflets were stripped off leaving behind their midribs each about 3 cm long. The main petiole was also trimmed down to about 3 cm length. These explants were set into Petri dishes, containing 1.5 % agar and the desired test compound. For distal applications the distal ends of the two debladed midribs were inserted into the agar, and for the proximal treatment the base of the main petiole was inserted into the agar. Between 16 and 20 abscission zones were employed per treatment. These sets were kept in darkness at 18° C. Counts of abscised petioles were made daily. All explants were tapped gently with a dissecting needle to dislodge ready pieces.

For simultaneous proximal and distal treatments, Petri dishes containing two thick blocks of agar were employed, and the proximal and distal ends were separately inserted into the agar blocks which then supplied the two desired auxin concentrations. The petiole explants were thus suspended horizontally between the blocks.

In the case of bean experiments the various auxins were employed as the aqueous solutions of their ammonium salts in agar. The use of ethyl alcohol as a solvent for auxins was avoided because pectin-methylesterase, which may be involved in the abscission reactions, is very sensitive to ethanol (5). Only 0.5 ml of concentrated NH_4OH was used to dissolve the auxin and the solution finally neutralized to pH 7.0. Ammonium hydroxide itself was found to have no

TABLE I

THE AVERAGE NUMBER OF DAYS FOR ABSCISSION FOR VARIOUS PAIRS OF COLEUS PETIOLES TREATED DISTALLY WITH VARIOUS CONCENTRATIONS OF NAPHTHALENEACETIC ACID

NAA	Leaf pair		
	3rd	4th	5th
ppm	*Days required for 100 % abscission*		
0	6.9 ± 0.21	6.3 ± 0.15	5.7 ± 0.26
0.1	6.1 ± 0.27	5.1 ± 0.21	4.6 ± 0.27
1	6.0 ± 0.21	5.9 ± 0.23	4.8 ± 0.20
10	7.5 ± 0.17	6.6 ± 0.22	5.7 ± 0.40
100	20.8 ± 0.85	11.2 ± 1.0	6.9 ± 0.14
1000	35.2 ± 1.5	15.5 ± 2.0	8.6 ± 0.72

Each item represents 10 petioles, reported with standard errors.

effect on abscission between 3×10^{-4} and 3×10^{-2} molar concentrations.

EXPERIMENTAL RESULTS

QUANTITATIVE EFFECTS: To determine the quantitative effects of auxin on abscission, various concentrations of auxin-lanolin mixture were applied on the distal tips of debladed Coleus petioles. The results of an experiment using naphthaleneacetic acid are presented in table I. Petiole abscission was clearly delayed by very high concentrations. Treatment with 1000 ppm (5.4×10^{-3} M) was enough to delay the abscission for one month in the youngest petioles. But it may be seen from all the three petiole pairs that the delaying effect could be shown only with paste concentrations of 10 ppm or more. With more dilute treatments, the petioles abscised sooner than the controls. Similar promotions and inhibitions were obtained with indoleacetic acid though at slightly higher concentrations.

To demonstrate the effectiveness of different auxin concentrations with the passage of time, a time curve

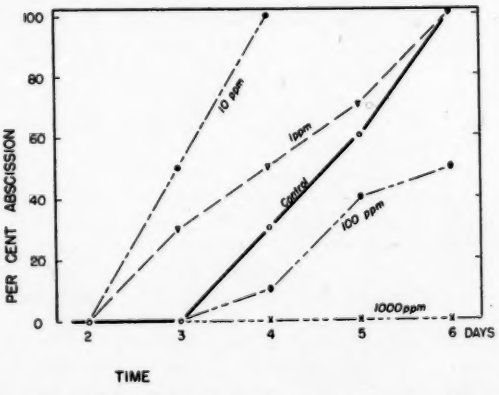

FIG. 1. The dual effect of NAA on the abscission of Coleus petioles, as a function of time after treatment. Leaf blades of the 3rd leaf pair were removed and auxin pastes applied to distal end at day 0.

is presented in figure 1. It appears that auxin applications at 1 and 10 ppm (5.4×10^{-6} and 5.4×10^{-5} M) caused a marked promotion of abscission, most evident from the 3rd to 5th day, whereas higher concentrations were inhibitory in their effects.

POSITIONAL EFFECTS: It has been suggested by Addicott and Lynch (1) that auxin may have entirely different effects upon abscission depending on the site of application. In order to evaluate quantitatively the differences in effects due to the application site, auxin solutions in agar were supplied from proximal and distal positions to bean petiole explants.

The data obtained from a typical bean petiole experiment with naphthaleneacetic acid are shown in figure 2. The graph shows the percentage of abscission 3 days after treatment with auxin at the proximal or at the distal end. Again it is evident that lower concentrations (1 and 10 ppm naphthalene

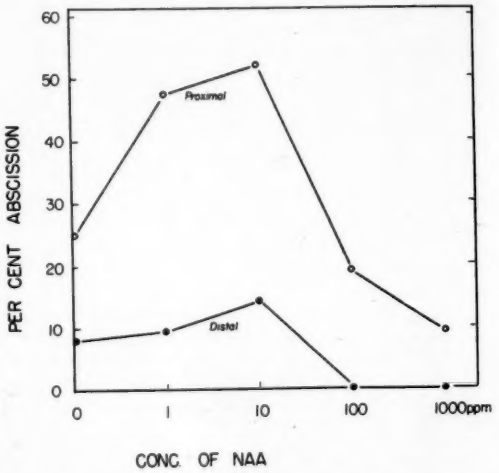

FIG. 2. The parallel abscission effects of various concentrations of NAA applied to the distal and to the proximal ends of bean petiole explants. Data for the 3rd day of treatment, 12 petiolules per treatment.

acetic acid, NAA) promoted abscission and higher concentrations prevented abscission to a large extent. This was true whether auxin was applied at distal or at proximal sites. Also it can be seen that in almost all the comparable treatments there was a higher percentage of abscission in proximal settings compared to distal ones. It may again be noted from this figure that though there is a quantitative difference between proximal and distal applications, still low auxin concentrations promoted and high concentrations inhibited abscission regardless of the site of application.

In order to give a general picture of the reversibility of auxin action with increasing concentrations, the means of three experiments on proximal treatment of bean petioles are plotted together in figure 3. It is evident that the abscission is stimulated by auxin treatment up to 10 ppm concentration above which the expected inhibition was obtained.

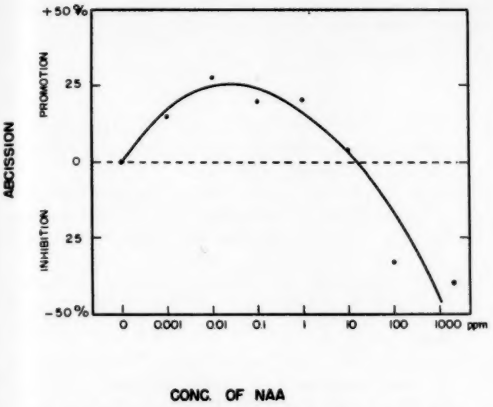

FIG. 3. The effects of NAA on abscission in bean petioles as a function of concentration. Data for the 3rd day of treatment from 3 experiments using proximal applications. Control values: 60, 62 and 65%, respectively.

AUXIN GRADIENT EFFECTS: In order to bring out evidence concerning whether it is the total amount of auxin or alternatively the auxin gradient that controls abscission, simultaneous applications with different auxin concentrations were made to the proximal and the distal ends of the bean petiole explants. In this experiment the ratio of proximal/distal auxin applied was kept constant either at 1/100 or at 100/1.

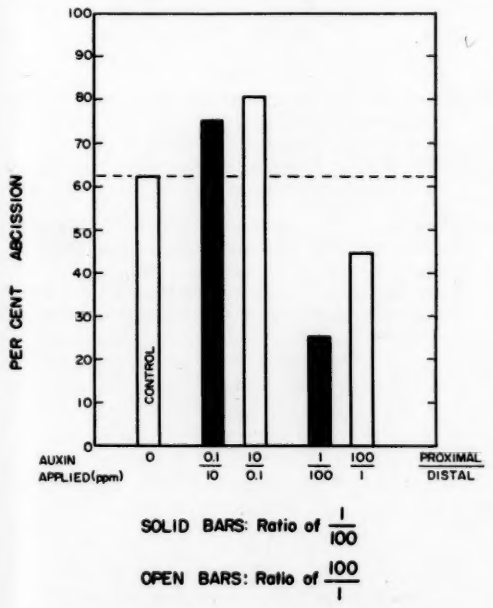

FIG. 4. The effects of artificially induced auxin gradients on abscission of excised bean petioles. Two concentrations of NAA were applied simultaneously at each end of the explants, and data reported for 5th day.

In this way an arbitrary gradient was imposed, but using different actual concentrations of auxin. Results are presented in figure 4. It may be seen that 10.1 ppm of NAA (10 ppm distal + 0.1 ppm proximal, or vice versa) whether applied in the ratio of 1/100 or 100/1 was always stimulatory to abscission. On the other hand, 101.0 ppm of the auxin (100 ppm + 1 ppm) proved inhibitory to abscission, no matter which way the gradient was established.

DISCUSSION

Perhaps the most striking finding in these experiments is the ability of auxins to promote abscission regardless of site of application. The possibility appears, thus, that auxins may not only prevent the abscission of plant organs when present in abundance, but further, when the auxin supply from an organ drops to a low level the process of abscission may actually be stimulated by the same substance which otherwise prevents abscission. Such a dual role of auxin in either promoting or inhibiting abscission is closely analogous to the dual effects of auxin in such phenomena as water uptake, respiration, protoplasmic streaming, and growth itself (8).

In studying the quantitative effects of auxins on the pedicels of apple, Barlow (3) found only an inhibition of abscission with distal auxin treatments. He did not observe any promotion of abscission even at very low auxin concentrations. It seems likely that the stimulatory effect of low concentrations may have been masked in his experiments by the high content of natural hormone already present in the fruit pedicels before treatment. Thus the effect of adding even small amounts was like that of a relatively high auxin concentration.

In the present studies it may be seen from figure 4 that higher concentrations such as 100 ppm applied on the proximal side were inhibitive of abscission. The same application on the distal side was more inhibitive than the proximal one. Such inhibitions by high auxin levels regardless of site of application can be seen also in figure 2. In earlier experiments in which rather large amounts were applied simultaneously to both proximal and distal sides Addicott and Lynch (1) also obtained only an inhibition of abscission no matter whether the proximal application was of lower or higher concentration than the distal one. It is clear now that higher concentrations of auxins when applied proximally can result in an inhibition of abscission only slightly less effectively than when applied distally.

The greater inhibition of abscission by distal compared to proximal treatments is probably a consequence of the predominantly polar translocation of auxin in leaf petioles (9, 2). It is logical to expect that a given application to the distal end would provide more auxin to the abscission zone than the same application to the proximal end (the physiological base).

Regarding the stimulation of abscission by low concentrations applied on the proximal side, it may be seen from the histogram in figure 4 as well as the gen-

eral curve (fig 3) that auxin was stimulatory to abscission in the range of 0.1 to 10.0 ppm. These observations call to mind the findings of Rossetter and Jacobs (14) who found that the intact leaves of Coleus speeded abscission of nearby debladed petioles. This stimulating effect of nearby leaves may have been a consequence of their providing a source of small amounts of auxin.

According to the gradient theory of Shoji et al (15) an auxin gradient between leaf blade and petiole, and not the absolute auxin content of the blade controls abscission. However, Addicott and Lynch (1) state that a simultaneous application of auxin to both ends of a petiole irrespective of the concentrations, resulted in the delaying of abscission. Similarly our observations with the simultaneous applications (fig 4) clearly indicate that the total amount of auxin applied was the controlling factor in the stimulation or inhibition of abscission and not the ratios of the auxin applications on the two sides. Thus from the above observations of the concentration effects and the direct test of the gradient theory, it appears that the total amount of auxin applied was the controlling factor in the stimulation or inhibition of abscission and not the auxin-gradient.

Summary

The relative effectiveness of different concentrations of naphthaleneacetic acid in controlling petiole abscission was tested at two sites of application, namely at distal and proximal positions. *Phaseolus vulgaris* L. var. Red Kidney and *Coleus blumei* Benth. var. Purdue Fern Marie were the plants utilized. Debladed petioles of Coleus were treated with lanolin paste of the auxin. Petiole explants obtained from first trifoliate leaves of bean were inserted in proximal and distal positions in agar plates containing the test compound in desired concentrations. The following conclusions were derived:

1. While high concentrations of auxin inhibit abscission, low concentrations have an opposite and promotive effect on abscission.

2. The differences in effects of proximal and distal applications appeared to be more of quantitative than qualitative differences.

3. The artificial induction of auxin gradients across excised petioles indicated that abscission was apparently controlled by the quantity of auxin and not by the gradient of auxin.

Literature Cited

1. Addicott, F. T. and Lynch, R. S. Acceleration and retardation of abscission by indoleacetic acid. Science 114: 688–689. 1951.
2. Avery, G. S., Jr. Differential distribution of a phytohormone in the developing leaf of Nicotiana and its relation to polarized growth. Bull. Torrey Bot. Club 62: 313–330. 1935.
3. Barlow, H. W. B. Studies in abscission. I. Factors affecting the inhibition of abscission by synthetic growth substances. Jour. Exptl. Bot. 1: 264–281. 1950.
4. Gardner, F. E. and Cooper, W. C. Effectiveness of growth substances in delaying abscission of Coleus petioles. Bot. Gaz. 105: 80–89. 1943.
5. Kertesz, Z. I. The Pectic Substances. Pp. 1–371. Interscience Publishers, Inc., New York. 1951.
6. Laibach, F. Wuchstoffversuche mit lebenden Orchideenpollinen. Ber. deut. bot. Ges. 51: 336–340. 1951.
7. La Rue, C. D. The effect of auxin on the abscission of petioles. Proc. Nat. Acad. Sci. 22: 254–259. 1936.
8. Leopold, A. C. Auxins and Plant Growth. Pp. 1–354. Univ. of Calif. Press, Berkeley, California. 1955.
9. Mai, G. Korrelationsuntersuchungen an entspreiteten Blattstielen mittels lebenden Orchideenpollinen als Wuchstoffquelle. Jahrb. wiss. Bot. 79: 681–713. 1936.
10. Mohl, H. von. Über den Ablosungsprocess säftiger Pflanzenorgane. Bot. Zeit. 18: 273–274. 1860.
11. Myers, R. M. Factors affecting the abscission of the leaves of *Coleus blumei*. Trans. Illinois State Acad. Sci. 32: 80–81. 1939.
12. Myers, R. M. The effect of heteroauxin on the development of debladed petioles of Coleus. Trans. Illinois State Acad. Sci. 33: 89–90. 1946.
13. Myers, R. M. Effect of growth substances on the abscis layer in leaves of Coleus. Bot. Gaz. 102: 323–338. 1940.
14. Rossetter, F. N. and Jacobs, Wm. P. Studies on abscission. The stimulating role of nearby leaves. Amer. Jour. Bot. 40: 276–280. 1953.
15. Shoji, K., Addicott, F. T., and Swets, W. A. Auxin in relation to leaf blade abscission. Plant Physiol. 26: 189–191. 1951.

CARBON DIOXIDE FIXATION BY ETIOLATED PLANTS AFTER EXPOSURE TO WHITE LIGHT[1,2]

N. E. TOLBERT AND F. B. GAILEY[3]

BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY, OAK RIDGE, TENNESSEE

Upon exposure of an etiolated plant to light, photochemical, biochemical, and physiological changes occur. Total chlorophyll synthesis and the conversion of protochlorophyll to chlorophyll a, have been studied extensively (10, 13). Gross physiological changes and cessation of elongation have been the subject of many investigations in plant physiology. Some of these processes are independent of chlorophyll synthesis and photosynthesis (22). A few changes in enzymatic activity during the greening of an etiolated plant have been reported (1, 8, 17).

Measurements of the formation of chlorophyll by the etiolated plant after exposure to light have been extensively investigated. Oxygen evolution by etiolated barley plants after they were placed in the light indicated that this process did not commence until the seedlings had been in the light 30 minutes (14). Experiments with etiolated oats also showed oxygen evolution after chlorophyll formation (6). Irving (9) reported that greening etiolated barley and bean seedlings did not fix CO_2 until they had developed a large part of their chlorophyll. Observations have now been made on the fixation of $C^{14}O_2$ and the products formed during increasing time intervals after an etiolated wheat plant has been placed in the light. The results indicate that for many hours after the start of illumination, there is a slow and irregular formation of some of the reactions in CO_2 fixation during photosynthesis.

PROCEDURE

Etiolated Thatcher wheat plants were grown at 24° C in sand with Hoagland nutrient solution. On the seventh day the plants were illuminated with between 600 and 1000 fc of continuous white light from 150-watt reflector flood bulbs. Water filters removed the heat. Fifteen leaves were taken for chlorophyll analyses (22) at hourly intervals after illumination began. At the same intervals but with different batches of wheat, the ability of the leaves to fix CO_2 was measured. For this latter test, one or two leaves were exposed in a small-scale photosynthesis chamber (7) for 10 minutes at 1000 fc of light to an atmosphere of air containing $C^{14}O_2$. The volume of the chamber was 100 ml into which was released 25 μc of C^{14} from $NaC^{14}O_3$ of a specific activity of 25 % C^{14} so that the final CO_2 concentration did not increase appreciably. During this period the temperature was kept at 23° C by submerging the apparatus in a water bath.

At the end of the 10-minute period, the C^{14}-exposed leaves were killed by maceration in boiling 30 % methanol. An aliquot was counted for total C^{14} fixation in the soluble extract. Another portion was subjected to paper chromatographic analysis, water-saturated phenol being used as the first solvent and butanol–propionic acid–water for the second development (3). Autoradiographs were made of each chromatogram, with No-Screen x-ray film to locate the radioactive compounds; the total counts per spot were determined with a G-M tube. This activity was then expressed as a percentage of the total activity on the chromatogram or as actual counts per second of the compound in one leaf. Since it was not practicable to start with an exact quantitative aliquot on each paper chromatogram, counts of radioactivity in the compounds among different experiments may be in error by as much as 15 %. The percentage distribution of radioactivity within one experiment should not be more variable than the counting errors.

In later experiments etiolated plants were exposed to light after first being sprayed with various compounds which might effect the greening process. In separate experiments, plants were sprayed with water, 0.3 M glucose, 0.3 M ribose, 0.1 M mixture of 20 % sedoheptulose and 80 % sedoheptulosan (designated sedoheptulose after its physiologically active component), or 0.1 M glycolic acid. The plants of each experiment were sprayed, while still in the dark, at 24, 22, 19, 16, and 0 hour intervals before illumination. Bottles for spraying indicator solutions on paper chromatograms were used. These conditions were chosen since the same procedure with glycolic acid has been shown to activate glycolic acid oxidase (17). Glycolic acid at pH 2.3 was used since these previous experiments have shown that the sodium salt of the acid when sprayed on leaves was not effective in activating glycolic acid oxidase. After exposure to light and just before the 10-minute photosynthesis test with $C^{14}O_2$, the leaf blades were cut off and washed briefly under running, distilled water to remove excess reagent. The leaves appeared normal and contained no visible bacterial or mold contamination from this treatment.

RESULTS AND DISCUSSION

Several light intensities were used in trial experiments to ascertain the most advantageous conditions for chlorophyll synthesis. These qualitative results indicated that light intensities around 1000 fc produce the most rapid greening; whereas, in very high light intensity from a photoflood bulb or sunlight, the plants greened very slowly. Of equal importance, etiolated leaves cut off at their base and placed in water turned green and fixed $C^{14}O_2$ at much slower rates than in experiments where the plant remained

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[3] Research Participant during Summer 1954. Present address: Biology Department, Berea College, Berea, Kentucky.

intact during the greening period. Racusen and Aronoff have shown that decapitated leaves incorporate amino acids into protein more slowly than in the intact plant (11), and thus the slower greening process in decapitated etiolated leaves in the present experiments may reflect less rapid protein synthesis for the formation of the chloroplast. However, cutting off the leaf for the final 10-minute photosynthesis experiment with $C^{14}O_2$ did not seem to affect the $C^{14}O_2$ fixation. Thus there are several differences between the present experiments and those of Irving (9): 1) The use of the whole etiolated plant rather than a cut-off top has been found necessary for satisfactory results; 2) more controlled and somewhat higher light intensities have been used; and 3) radioactive $C^{14}O_2$ has permitted measurements of rate of fixation and products formed. It has not been possible to ascertain the effect of unlabeled CO_2 produced inside the cell by respiration on the rate of fixation of external radioactive $C^{14}O_2$. However, the absence of C^{14} in the products of the path of carbon in photosynthesis for several hours of greening would indicate no photosynthetic fixation, and permits qualitative measurements on the formation of this process.

Chlorophyll Formation and CO_2 Fixation Rates: Figure 1 shows chlorophyll content and total $C^{14}O_2$ fixation at various times after etiolated leaves were first exposed to light. A very small amount of chlorophyll was present after the plants had been in the light for 1 hour, owing to the rapid light-catalyzed conversion of protochlorophyll to chlorophyll, but large amounts were not produced for 2 to 3 hours. The rate of chlorophyll production was most rapid from about the third to the twelfth hour of light. After this period, the amount of chlorophyll in the plant increased more slowly, on wet weight basis, for about 20 hours. After 1.5 days of continuous light, the now fully green plant contained a normal amount of chlorophyll.

The rate of $C^{14}O_2$ fixation was very slow for about 1 to 2 hours after rapid chlorophyll production had started, i.e., after about 5 hours of continuous light. The amount fixed before this time, as measured accurately by the isotope method, amounted to less than 0.1 % of that fixed by the leaves after greening. Thus when these etiolated plants were placed in the light, several hours were required for the rate of chlorophyll synthesis to become significant, and then 1 to 2 more hours of light before photosynthesis commenced, as measured by $C^{14}O_2$ fixation. Total fixation of $C^{14}O_2$ began to increase at a rapid, linear rate after photosynthetic fixation began, around the fifth hour of light, and so continued to increase during the next 24 hours or longer. After about 32 hours the plant was fully green and fixed CO_2 at a rate equal to that of a normal plant. Almost all the C^{14} was fixed into the soluble extract during the 10-minute photosynthesis test period with $C^{14}O_2$ by the greening plants and the data reported are values for the activity in the soluble fraction only.

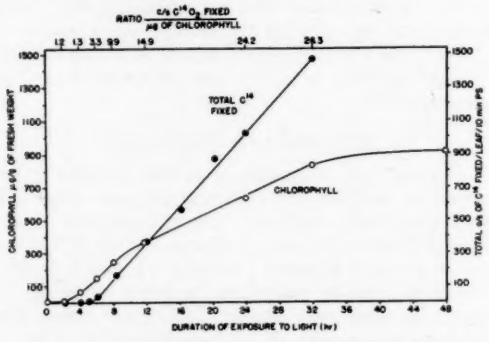
RATIO c/s C14O2 FIXED / µg OF CHLOROPHYLL
1.2 1.3 3.3 9.9 14.9 24.2 28.3
TOTAL C14 FIXED
CHLOROPHYLL
CHLOROPHYLL, µ g/g OF FRESH WEIGHT
TOTAL c/s OF C14 FIXED/LEAF/10 min PS
DURATION OF EXPOSURE TO LIGHT (hr)

Fig. 1. Total chlorophyll and rate of $C^{14}O_2$ fixation during greening.

Smith (14) noted that oxygen evolution during greening in etiolated barley seedlings began within 30 minutes which indicated that a Hill reaction was functioning in the whole leaves. At that time the chlorophyll present was chiefly the small amounts formed by the initial photochemical conversion of protochlorophyll to chlorophyll. Evolution of oxygen from oat leaves soon after exposure to light has also been reported (6). CO_2 fixation, however, appears to commence later if comparison between these different monocotyledonous plants is reasonable. Such a comparison seems valid in that the rate and time of chlorophyll synthesis were similar.

The ratio of the rate of $C^{14}O_2$ fixation to total chlorophyll is given across the top of figure 1 for each period of analysis after the etiolated plants were placed in the light. Data on amount of chlorophyll formed has been reported as microgram per gram of fresh tissue weight, in conformance with similar figures in the literature. The average weight of one leaf (< 100 mg) for each analysis was determined for calculating this ratio, since the leaves for $C^{14}O_2$ fixation experiments were not weighed, in order to facilitate rapid killing for subsequent chromatographic analysis of the products. The ratios indicate that with increasing time in 1000 fc of light the chlorophyll present in the greening wheat became much more efficient in promoting CO_2 fixation. This ratio, calculated for these particular experimental conditions, and C^{14} specific activity could vary with changes in light intensity, temperature, and CO_2 partial pressure. Increasing light intensity and CO_2 pressure might tend to increase the difference in this ratio since the very small fixation rate in the slightly green plant should not be limited by these factors. However, further studies based on Blackman's principle of a rate-limiting step in the multiple process of photosynthesis should aid in determining which portions of photosynthesis are rate limiting during various times in the greening process.

Products of $C^{14}O_2$ Fixation: The products formed by fixation of $C^{14}O_2$ for a 10-minute period at the end of each hour after the plants had been placed in white light were analyzed by paper chromatography. Figures 2 A and B are autoradiographs of

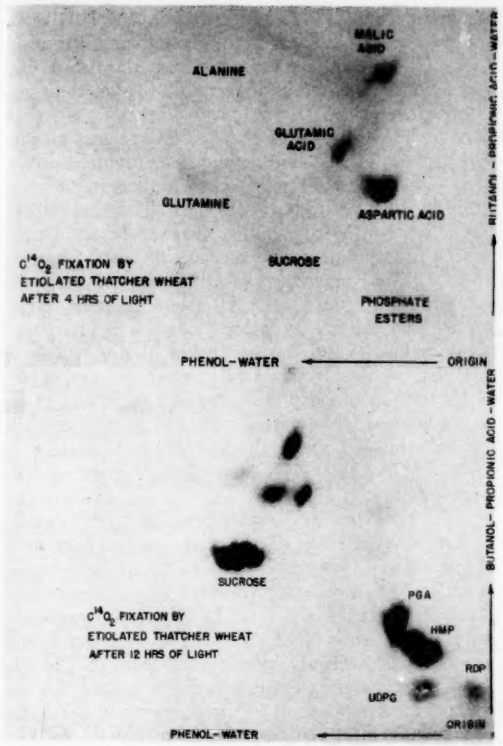

FIG. 2. Products from $C^{14}O_2$ fixation: A (*above*), after 4 hrs of light; B (*below*), after 12 hrs of light.

the chromatograms of leaves which had had 4 hours and 12 hours of light, respectively. C^{14} activity in each compound was counted after detection on the chromatograms by autoradiography, and is expressed in figure 3 as a percentage of the total C^{14} fixed during that period. During the first 4 hours after the etiolated plants were placed in the light, the only three products to be labeled by $C^{14}O_2$ in significant amounts were malic, aspartic, and glutamic acids. The curve for percentage of C^{14} in glutamic acid (not shown) was similar to that for aspartic acid; however, there was only about one-half as much label in glutamic acid. These products represent respiratory fixation. As will be shown later, small amounts of C^{14} in phosphoglyceric acid (PGA) and sugar phosphates were also detectable by the isotope method after 4 hours of light. But the amount of this photosynthetic fixation was not significant even though the plants were in the light and had formed appreciable amounts of chlorophyll by this time.

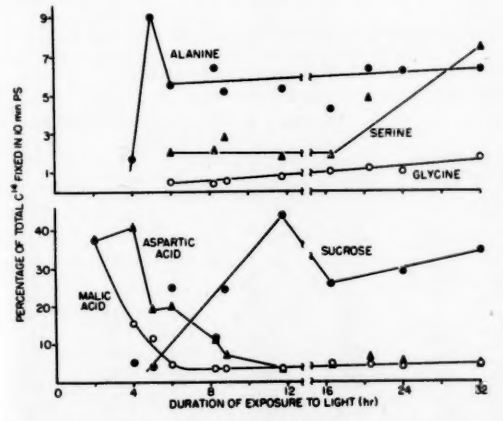

FIG. 3. Percentage of total C^{14} fixed in certain compounds with increasing lumination periods.

As the plant greened during 2 to 8 hours of light, the total C^{14} incorporated into malic, aspartic, and glutamic acids increased manyfold as chlorophyll was produced (table I). The great increase in the amount of total C^{14} in the respiratory products was perhaps stimulated by the photosynthetic reducing power being produced by the newly formed chlorophyll. The early increase was probably not caused by an increase in the pool size of newly formed intermediate compounds in the photosynthetic cycle, since these compounds had not yet accumulated in appreciable amounts.

TABLE I

RELATIVE AMOUNTS OF C^{14} INCORPORATED IN MALIC, ASPARTIC, AND GLUTAMIC ACIDS

DURATION OF LIGHT *	TOTAL FIXATION PER LEAF PER 10 MIN		
	MALIC	ASPARTIC	GLUTAMIC
hr	*c/s*	*c/s*	*c/s*
2	>1	>1	<1
4	4	10	3
6	7	29	6
8	22	71	31
9	54	97	45
12	56	50	38
24	164	232	158
32	268	334	46

* For chlorophyll concentration at each of these periods, see figure 1.

The first product, other than the respiratory acids, to become labeled with C^{14} after the etiolated wheat plants had been placed in the light was a small amount of PGA after about 4 hours of light (tables IV, V, VI). At the fifth hour, PGA and alanine contained an abnormally high percentage of the total C^{14} fixed. Figure 3 shows the percentage of C^{14} in alanine at this time. The C^{14} activity in alanine was probably derived from PGA through pyruvic acid, though the intermediate C_3 compounds did not accumulate in appreciable amounts. It appears that the plants, at this stage of greening, were able to reduce little of the PGA to the aldehyde level and then convert it to carbohydrate. With plants exposed to white light for longer periods of time, the percentage of the total C^{14} fixed in the alanine dropped to a smaller, but there-

after constant, amount. However, the total radioactivity in the C_3 compounds continued to increase with increasing rate of $C^{14}O_2$ fixation.

These results suggest that after 5 hours of light, the plant was able to commence slow photosynthetic carboxylation to form PGA, of which part was converted to alanine. Several explanations are then possible to explain the accumulation of alanine and PGA, with little C^{14} activity appearing in the sugars. At that stage, the plant may not have had sufficient reducing power or active enzymes to reduce rapidly PGA to hexose. On the other hand, saturation of the alanine and PGA pools may have been necessary for equilibria favoring synthesis of sucrose. In either case, this condition seemed to exist for an interval of 1 to 2 hours at about the fifth hour after etiolated Thatcher wheat had been placed in white light.

After exposure of the plant to light for longer than 5 hours, quantities of C^{14} began to appear in the sugar phosphates and sucrose. The percentage of C^{14} going into sucrose and sugar phosphates, as reflected by sucrose (fig 3), increased rapidly at this time, to reach a high and relatively constant maximum percentage value after the plants had been illuminated for 9 to 12 hours. Longer periods of greening seemed to have no further effect on the percentage of the fixed C^{14} which appeared in sucrose. However, it is important to note that after this period of light, the total C^{14} fixed was still only 15 to 20 % of that of a fully greened plant after 32 hours of light (fig 1). The data indicate that by this time the reactions in the reduction of PGA were functioning at about the equilibrium conditions of normal steady-state photosynthesis. The increase in total fixation rate after 12 hours must be the result of the increasing rate of other, more slowly developing processes; such as formation of chlorophyll and of the CO_2 acceptor, which is ribulose diphosphate (RDP) or related to it. On the basis of the increasing chlorophyll efficiency ratio for total CO_2 fixation with further greening, still other factors probably exist which limit photosynthesis after only 12 hours of greening.

Formation of Serine and Glycine: Serine and glycine acquired C^{14} from $C^{14}O_2$ during greening of the etiolated plant on a different time sequence from that of alanine (fig 3). PGA and alanine became radioactive during the 10-minute $C^{14}O_2$ fixation period in leaves that had greened for 4 and 5 hours but glycine and serine were not labeled. In fact, about 20 hours of light were required for a large amount of C^{14} to be incorporated into serine. During photosynthetic fixation experiments with $C^{14}O_2$ by green barley or wheat plants, glycine and serine are always labeled with a large portion of the C^{14} fixed (5, 16). It has been assumed that these two amino acids were labeled quickly during short-time photosynthesis experiments because they were end products of a short side reaction from the carbon cycle of photosynthetic fixation (12, 18, 20, 21). At least two pathways have been proposed for glycine and serine formation in plants; namely, the formation of serine from PGA, or their formation from glycolic and glyoxylic acids. Glycolic acid is readily converted to glycine and serine in plants (12, 18) and should arise from the two carbon fragments by the action of transketolase on the sugar phosphates formed during photosynthesis. Furthermore, it has been shown that a key enzyme, glycolic acid oxidase, of this side reaction is activated in etiolated plants during the greening process in order to establish this pathway (17). For Thatcher wheat, this activation period required about 20 hours of light. In the present experiments, again with Thatcher wheat, a rapid movement of C^{14} into serine was also not established until after 20 hours of light, which implies that the major pathway for the in vivo incorporation of C^{14} into serine during photosynthesis in this plant is via glycolic acid. Serine did not appear to be formed directly from triose and PGA, as has been proposed for soybeans (20), since it was not substantially labeled during the first 20 hours of greening, while alanine and PGA were being produced with large amounts of C^{14}.

CO_2 Fixation by Leaves Sprayed with Various Compounds: A more detailed analysis has been made of the organic phosphate esters of the path of carbon in photosynthesis during the first hours the etiolated plant acquired the ability to fix $C^{14}O_2$ photosynthetically. Also, amounts of several of these intermediates were raised artificially by spraying them on the etiolated plant before exposure to light. Since the percentage incorporation of C^{14} in sucrose had not varied significantly after the plants had been in the light for 9 to 12 hours, in these experiments data on plants in the light for longer than 8 hours were not kept except for one 25-hour exposure.

Total C^{14} fixed by approximately equal leaf samples sprayed with water in the dark 24 hours (used as controls) did not vary significantly from that fixed by unsprayed plants (table II). Feeding glucose to the etiolated plant prior to the experiment gave some increase in the rate of chlorophyll synthesis but a sub-

Table II

Influence of Spraying Various Substrates on Etiolated Wheat Leaves Prior to Illumination on Rate of Photosynthesis

Duration of light	Solution sprayed on leaves prior to light exposure				
	Water *	Glucose	Ribose	Sedoheptulose	Glycolic acid
hr	*c/s*	*c/s*	*c/s*	*c/s*	*c/s*
2	13	4	8	3	10
3	8	48	75	4	12
4	54	92	350	11	23
5	103	274	253	57	151
6	238	230	172	180	374
7	300	527	303	290	250
8	725	510	478	370	550
25	1042	1800	1471	1350	1400

* Total C^{14} fixed during a 10-min test period with $C^{14}O_2$ into the water soluble extract from 2 leaves.

stantially higher rate of CO_2 fixation during the critical time after 3 to 5 hours of light. Ribose fed to the etiolated plant stimulated CO_2 fixation even more. For both sugars, there still remained a 2-hour lag in light before CO_2 fixation began. In both cases, but not for the water-sprayed control, after 6 hours of light there was a great falter in the rate of increase of CO_2 fixation by the plant as if some precursor or factor had been exhausted, and its synthesis was rate limiting for a few hours thereafter. One explanation for this may be that the presence of the free sugars in large amounts in the leaf resulted in an unusual demand on adenosine triphosphate (ATP) for their phosphorylation before they could be used for the plant's growth. Thus much of the ATP produced by the photosynthesis process (15) may not have been available at this time for regeneration of the intermediates for photosynthesis.

Sedoheptulose sprayed on the etiolated leaves in the dark inhibited CO_2 fixation rather than stimulating it. These results were not expected since sedoheptulose phosphate is intimately involved in the metabolic processes of photosynthesis (4). Since 80 % of this sugar was present in the spray as its anhydride, and since this anhydride is not metabolized by barley leaves (19), there is a possibility that the inhibition of the path of carbon of photosynthesis during greening may be due to this unnatural form of the seven carbon sugar.

Glycolic acid had no effect on the rate of CO_2 fixation during the first hours the leaves were in the light. Glycolic acid, when applied in exactly the same manner, has been shown to activate glycolic acid oxidase in this plant (17). Therefore, this enzyme does not appear to be rate limiting in photosynthesis during greening, but rather is a side reaction from this process.

After illumination for about 1 day, all the plants sprayed with the various compounds had a higher rate of $C^{14}O_2$ fixation than the water-sprayed control. Compounds sprayed on the plant were then perhaps being utilized to stimulate growth of the plant.

In table III are tabulated the amounts of radioactive carbon fixed in sucrose and the organic phosphate esters located on the chromatograms between PGA and the origin. These compounds represent a major fraction of the total C^{14} fixed and include all the sugar phosphates and PGA which are the active constituents of the path of carbon in photosynthesis. Glucose-, and especially ribose-treated plants, had a stimulated sucrose synthesis after 4 hours and again after 25 hours of illumination, in comparison to the water controls. The data indicate that the increased total fixation (table II) was a result of photosynthesis and not of greatly enhanced respiratory fixation.

Composition of Organic Phosphate Esters: The first product of photosynthetic $C^{14}O_2$ fixation is carboxyl-labeled PGA and the immediate precursor for this carboxylation is probably the pentose sugar, ribulose diphosphate (2). In the greening, etiolated leaf, the pool size of this precursor should be vanishingly small or nonexistent. Analysis of the plant tissue by the chromatographic solvents employed gave poor separation of the phosphate esters. These areas on the chromatogram have been designated according to their major component as PGA, HMP (hexose monophosphate), UDPG (uridine diphosphate–glucose), and RDP (fig 2 B). A more detailed analysis of these phosphate ester areas has been made by eluting each phosphate area separately and hydrolyzing the ester with a crude phosphatase preparation (Polidase S). Rechromatography of the organic moiety containing the C^{14} gave good separation and provided identification of the original phosphate esters in each area. In tables IV, V, and VI, the yields of such dephosphorylated products have been given relative scores to indicate the order in which these key esters acquire C^{14} from $C^{14}O_2$ during the greening period.

Results in table IV are for the Thatcher wheat

Table III

Sucrose and Organic Phosphate Esters Formed During Photosynthesis by Greening Wheat Leaves

Duration of light	Reagent sprayed on leaves before experiment									
	Water		Glucose		R'bose		Sedoheptulose		Glycolic acid	
	Sucrose	Phosphates *	Sucrose	Phosphates	Sucrose	Phosphates	Sucrose	Phosphates	Sucrose	Phosphates
hr	*c/s*	*c/s*	*c/s*	*c/s*	*c/s*	*c/s*	*c/s*	*c/s*	*c/s*	*c/s*
2	Trace	<1	0	Trace	Trace	1	0	Trace	Trace	<1
3	Trace	<1	4	8	6	14	Trace	Trace	<1	<1
4	4	10	8	13	..	..	1	3	2	4
5	8	25	42	79	42	84	4	13	20	34
6	50	54	46	80	36	37	11	31	38	88
7	42	68	94	157	34	67	33	61	49	56
8	140	131	136	181	88	112	33	77	107	120
25	217	143	705	337	601	302	334	138	443	296

* Phosphates include the C^{14} in PGA, HMP, UDPG, and RDP. A more detailed analysis of these areas is given in tables IV, V and VI.

TABLE IV

COMPOSITION OF PHOSPHATE ESTERS FORMED DURING 10-MIN PHOTOSYNTHESIS BY ETIOLATED WHEAT LEAVES AFTER 24-HR SPRAYING WITH WATER AND THEN EXPOSURE TO WHITE LIGHT

DURATION OF LIGHT AND PHOSPHATE AREAS	HYDROLYSIS PRODUCTS *					
	GLYCERIC ACID	GLUCOSE	FRUCTOSE	SEDOHEPTULOSE	RIBULOSE	RIBOSE
2 hr						
Total area	Trace	Trace	0	0	0	0
3 hr						
PGA	+	0	0	0	0	0
HMP	0	Trace	Trace	0	0	0
UDPG ...	0	Trace	0	0	0	0
4 hr						
PGA	++	Trace	Trace	0	0	0
HMP	Trace	++	+	Trace	0	0
UDPG ...	0	+	0	0	0	0
RDP	0	Trace	Trace	Trace	0	0
5 hr						
PGA	++	Trace	Trace	0	0	0
HMP	Trace	++	+	Trace	0	0
UDPG ...	0	+	0	0	0	0
RDP	0	Trace	+	Trace	Trace	Trace
6 hr						
PGA	+++	Trace	0	0	0	0
HMP	Trace	++	+	Trace	0	0
UDPG ...	0	+	0	0	0	0
RDP	0	Trace	+	Trace	Trace	Trace
25 hr						
PGA	+++++	Trace	+	0	0	0
HMP	+	+++	++	++	0	0
UDPG ...	0	++	0	0	0	0
RDP	Trace	Trace	Trace	Trace	Trace	Trace

* The C^{14} labeled PGA, HMP, UDPG and RDP areas each were eluted separately from the original paper chromatogram, enzymatically hydrolyzed with Polidase S, the products rechromatographed, and autoradiograms made of new chromatograms. These autoradiographs were scored by visual observation while comparing whole sets of chromatograms. The total C^{14} in the combined organic phosphate areas before hydrolysis are given in table III.

leaves that had been sprayed with water and serve as controls for tables V and VI. After the etiolated plants had been in the light for 3 hours, detectable C^{14} activity was present in PGA, though this was but a small percentage of the total fixed into respiratory products. A vanishingly small trace of activity (too small to count) could be detected by autoradiography in glucose and fructose from the HMP and UDPG areas. The results indicate that the PGA pool had built up first. After 4 hours of light the plant can reduce a little $C^{14}O_2$ to the hexose sugar phosphates but the predominant fixation was still into malic, aspartic, and glutamic acids. The appearance of C^{14} in the hexoses indicates some reversal of the Embden-Meyerhof pathway at this stage.

The 10-minute exposure of $C^{14}O_2$ used in these experiments would be considered so long a labeling period in photosynthesis with a fully green leaf that about uniform distribution of C^{14} in the carbon atoms of all the sugars would be expected. Therefore the appearance of significant amounts of C^{14} in PGA alone over a 10-minute period represents a tremendous change in rate.

Only traces of C^{14} activity were present in RDP from the 5th- to the 25th-hour analyses after illumination of the leaves. Sedoheptulose phosphate did not appear to accumulate significant amounts of label until almost a day after the etiolated plants were placed in the light. This suggests that one reason for the lag in CO_2 fixation during the greening period and for the low efficiency of the newly formed chlorophyll was the failure of the plant to drive the photosynthetic cycle at a rate sufficiently high to regenerate enough RDP. The reversal of the Embden-Meyerhof pathway seemed to have been functioning satisfactorily, probably because many of its enzymes had been involved in the growth of the etiolated seedling.

It is impossible to determine by these data alone whether the absence of active enzymes in the regeneration of RDP may have been responsible for slow recycling of the C^{14} to ribulose diphosphate. The very short lag phase in CO_2 fixation (seconds to a few minutes) by a green plant, when transferred from dark to light, should be compared to the lag in photosynthesis of over 24 hours during the greening of the etiolated plant. The length of the induction period in photosynthesis by a fully green plant may be a measure of the rapidity with which the plant can

TABLE V

COMPOSITION OF PHOSPHATE ESTERS FORMED DURING 10-MIN PHOTOSYNTHESIS BY ETIOLATED WHEAT LEAVES AFTER 24-HR SPRAYING WITH GLUCOSE AND THEN EXPOSURE TO WHITE LIGHT

DURATION OF LIGHT AND PHOSPHATE AREAS	HYDROLYSIS PRODUCTS					
	GLYCERIC ACID	GLUCOSE	FRUCTOSE	SEDOHEPTULOSE	RIBULOSE	RIBOSE
2 hr						
Total areas	Trace	0	0	0	0	0
3 hr						
PGA	++	Trace	+	0	0	0
HMP	Trace	++	+	Trace	0	0
UDPG ...	0	+	Trace	Trace	0	0
4 hr						
PGA	+++	0	Trace	0	0	0
HMP	Trace	++	+	Trace	0	0
UDPG ...	0	++	Trace	Trace	0	0
5 hr						
PGA	+++++	Trace	Trace	0	0	0
HMP	+	+++	++	+	0	0
UDPG and RDP ...	Trace	++	Trace	Trace	Trace	Trace
6 hr						
PGA	+++++	Trace	Trace	0	0	0
HMP	+	+++	++	+	0	0
UDPG ...	0	++	Trace	0	0	0
RDP	0	Trace	Trace	Trace	+	Trace
25 hr						
PGA	++++	Trace	Trace	0	0	0
HMP	+	+++	++	+	0	0
UDPG ...	Trace	+++	0	0	0	0
RDP	Trace	++	Trace	Trace	++	+

TABLE VI

COMPOSITION OF PHOSPHATE ESTERS FORMED DURING 10-MIN PHOTOSYNTHESIS BY ETIOLATED WHEAT LEAVES AFTER 24-HR SPRAYING WITH RIBOSE AND THEN EXPOSURE TO WHITE LIGHT

DURATION OF LIGHT AND PHOSPHATE AREA	HYDROLYSIS PRODUCTS					
	GLYCERIC ACID	GLUCOSE	FRUCTOSE	SEDOHEPTULOSE	RIBULOSE	RIBOSE
2 hr						
PGA	+	+	Trace	0	0	0
UDPG ...	0	Trace	0	0	0	0
3 hr						
PGA	++	Trace	+	Trace	0	0
HMP	Trace	++	+	+	0	0
UDPG ...	0	+	Trace	0	0	0
RDP	0	Trace	Trace	Trace	0	0
4 hr						
PGA	++	Trace	+	Trace	0	0
HMP	Trace	++	+	Trace	0	0
UDPG and RDP ...	0	+	0	0	0	0
5 hr						
PGA	+++	0	0	0	0	0
HMP	+	+++	++	+	0	0
UDPG and RDP ...	Trace	++	+	Trace	Trace	0
6 hr						
PGA	+++	0	0	0	0	0
HMP	0	++	++	+	0	0
UDPG ...	0	+	0	0	0	0
RDP	0	Trace	+	Trace	Trace	Trace
25 hr						
PGA	+++++	+	++	+	0	0
HMP	++	+++++	+	++	0	0
UDPG ...	Trace	++	0	0	0	0
RDP	Trace	+	Trace	Trace	++	+

build up the pool size of carbon compounds in photosynthesis provided all the enzymatic processes are present. The 24-hour lag in photosynthesis and production of significant RDP by a greening etiolated plant, therefore, can hardly be explained by the slow accumulation of significant pools of these compounds. Rather, it would appear that synthesis of adequate amounts of enzymes to catalyze some of these reactions might be a limiting factor.

In the area of the sugar phosphate esters there was one unknown with an R_f of 0.2 in both the solvents used in chromatography. This unknown was not hydrolyzed by Polidase S. Its pool size with respect to total C^{14} became constant after the etiolated leaves had had only 4 hours of light. Thus the rate of labeling of this unknown was like that for PGA and alanine at the fifth hour of greening, except that it was present in rather small amounts and as the plant greened further, more total C^{14} was not incorporated into the compound. In these respects it was unique among the compounds being labeled by the $C^{14}O_2$.

When the etiolated leaves were sprayed with glucose prior to illumination, the rate and amount of $C^{14}O_2$ incorporation into PGA and the hexose were stimulated (table V). However, detectable label in pentose phosphates did not occur in the leaves until after they had been exposed to the same long period of light as required by the water-sprayed controls. Leaves sprayed with ribose prior to the light exposure had an even faster and more diversified buildup of the organic phosphate esters of photosynthesis (table VI). In these leaves, there were measurable amounts of PGA and hexose phosphates after only 2 hours of light; after 3 hours of light, considerable sedoheptulose phosphate was present, although it did not appear with the water controls until the fifth hour, and not in this amount until the twelfth hour of light. Nevertheless, RDP still showed the same small pool size at these times. This may be caused by some limiting step unaffected by the excess ribose present; or the large pool of fed and unlabeled pentose may prevent, by mass action, rapid recycling of the fixed $C^{14}O_2$ through RDP. This would have resulted in all the newly formed C^{14}-labeled PGA being used for hexoses and sucrose synthesis. However, after the plants had been in the light for 1 day, substantial RDP was present, which probably reflects the stimulated total CO_2 fixation by plants fed this sugar.

In table VII are summarized the relative amounts of C^{14} appearing during the 10-minute photosynthesis period with $C^{14}O_2$ in the products of an etiolated Thatcher wheat plant after exposure to 3 hours of light. At this time, C^{14} label could be detected in the first photosynthetic fixation products, although the amount fixed in them was not an appreciable percentage of the total fixation. Prior spray feeding of either glucose or ribose solutions to the etiolated leaves in the dark stimulated C^{14} fixation into PGA, glucose and fructose phosphates, and sucrose, but not into RDP.

Since the unique carboxylation reaction of photosynthesis utilizes RDP, the production of C^{14}-labeled PGA, hexoses, and sucrose without producing detectable label in the pentoses or RDP during the early phases of the greening process indicate that the pentoses were present in exceedingly small and rate-limiting amounts. As expected, feeding ribose to the etiolated plant resulted in a large increase in photosynthetic fixation at these times, since the plant should be able to convert it to RDP. In an etiolated plant not

TABLE VII

RELATIVE AMOUNTS OF C^{14}-LABELED PRODUCTS FROM 10-MIN PHOTOSYNTHESIS BY ETIOLATED WHEAT AFTER EXPOSURE TO 3 HR OF LIGHT

PRODUCTS	SOLUTIONS SPRAYED ON LEAVES PRIOR TO LIGHT EXPOSURE				
	WATER	GLUCOSE	RIBOSE	SEDOHEPTULOSE-SEDOSAN	GLYCOLIC ACID
Aspartic acid .	+++	++++	++++	+++	+++
Malic acid ...	++	+++	+++	++	++
PGA area	+	++	++	0	+
HMP area ...	Trace	+++	++++	Trace	+
UDPG area ..	Trace	+	+	0	Trace
Sucrose	Trace	+++	+++	Trace	+

sprayed with ribose, other sources for the RDP must be available for the first carboxylations, such as from the alternate glucose metabolism pathway. Afterward, the pool size of RDP remained very small, with the result that none had been detected in these experiments. An explanation has not been found for the formation of a large pool of hexoses and sucrose by the etiolated plants after 12 hours in the light while not converting them to RDP. At this time the plants were quite green, but the total photosynthetic CO_2 fixation was still relatively low. One explanation which needs further testing is that of slow enzymatic adaptation for RDP synthesis.

Summary

Etiolated Thatcher wheat plants were exposed to 1000 fc of white light and at hourly intervals afterward determinations were made of total chlorophyll, rate of $C^{14}O_2$ fixation, and the C^{14} products which were formed. Continuous, rapid chlorophyll synthesis began after 2 to 3 hours of light. During the first 4 hours of light, C^{14} was fixed at a low rate into malic, aspartic, and glutamic acids. After 4 and 5 hours of light, PGA and alanine were labeled, and after 5 to 6 hours of light C^{14} appeared in hexose phosphates and sucrose at an increasing rate. At the beginning of photosynthetic fixation there was a period during which C^{14} accumulated in PGA and alanine without being further reduced to hexoses in large amounts. After 9 to 12 hours of light, the percentage of the total C^{14} fixed which was incorporated among PGA, sucrose, and hexose phosphates, was constant, but only at one-quarter the rate of a normally green plant.

A limiting factor during this greening process was the availability of RDP, which may have been partially limited by slow enzymatic adaptation in the plant. The effect on the subsequent greening process of spraying glucose, ribose, sedoheptulose-sedoheptulosan, and glycolic acid on the etiolated plants in the dark prior to the exposure to light was studied. Ribose, and to a lesser extent, glucose, stimulated CO_2 fixation during the first hours of greening, but the amount of RDP present still appeared to be a rate-limiting factor. Glycolic acid was without effect and sedoheptulose-sedoheptulosan was inhibitory to the formation of an active photosynthetic cycle.

Label did not appear in large amounts in serine until after the period for activation of glycolic acid oxidase. This indicated that the major pathway for serine synthesis during photosynthesis was via the glycolic acid pathway.

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Literature Cited

1. Appleman, D. Catalase-chlorophyll relationship in barley seedlings. Plant Physiol. 27: 613–621. 1952.
2. Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. Z., Wilson, A. T., and Calvin, M. The path of carbon in photosynthesis. XXI. The cyclic regeneration of carbon dioxide acceptor. Jour. Amer. Chem. Soc. 76: 1760–1770. 1954.
3. Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A., and Stepka, W. The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. Jour. Amer. Chem. Soc. 72: 1710–1718. 1950.
4. Benson, A. A., Bassham, J. A., Calvin, M., Hall, A. G., Hirsch, H. E., Kawaguchi, S., Lynch, V., and Tolbert, N. E. The path of carbon in photosynthesis. XV. Ribulose and sedoheptulose. Jour. Biol. Chem. 196: 703–716. 1952.
5. Benson, A. A. and Calvin, M. The path of carbon in photosynthesis. VII. Respiration and photosynthesis. Jour. Exptl. Bot. 1: 63–68. 1949.
6. Blaauw-Jansen, G., Komen, J. G., and Thomas, J. B. On the relation between the formation of assimilatory pigments and the rate of photosynthesis in etiolated oat seedlings. Biochim. Biophys. Acta 5: 179–185. 1950.
7. Burris, R. H., Wilson, P. W., and Stutz, R. E. Incorporation of isotopic carbon into compounds by biosynthesis. Bot. Gaz. 111: 63–69. 1949.
8. Galston, A. W., Bonner, J., and Baker, R. S. Flavoprotein and peroxidase as components of the indoleacetic acid oxidase system of peas. Arch. Biochem. 42: 456–470. 1953.
9. Irving, A. A. The begining of photosynthesis and the development of chlorophyll. Annals Bot. 24: 805–819. 1910.
10. Koski, V. M., French, C. S., and Smith, J. H. C. The action spectrum for the transformation of protochlorophyll to chlorophyll a in normal and albino corn seedlings. Arch. Biochem. 31: 1–17. 1951.
11. Racusen, D. W. and Aronoff, S. Metabolism of soybean leaves. VI. Exploratory studies in protein metabolism. Arch. Biochem. Biophys. 51: 68–78. 1954.
12. Schou, L., Benson, A. A., Bassham, J. A., and Calvin, M. The path of carbon in photosynthesis. XI. The role of glycolic acid. Physiol. Plantarum 3: 487–495. 1950.
13. Smith, J. H. C. Protochlorophyll, precursor of chlorophyll. Arch. Biochem. 19: 449–454. 1948.
14. Smith, J. H. C. The development of chlorophyll and oxygen-evolving power in etiolated barley leaves when illuminated. Plant Physiol. 29: 143–148. 1954.
15. Strehler, B. L. Photosynthesis-energetics and phosphate metabolism. In: Phosphorus Metabolism, W. D. McElroy and B. Glass, ed. Vol. II, Pp. 491–502. Johns Hopkins Press, Baltimore. 1952.
16. Tolbert, N. E. Formic acid metabolism in barley leaves. Jour. Biol. Chem. 215: 27–34. 1955.
17. Tolbert, N. E. and Cohan, M. S. Activation of glycolic acid oxidase in plants. Jour. Biol. Chem. 204: 639–648. 1953.
18. Tolbert, N. E. and Cohan, M. S. Products formed from glycolic acid in plants. Jour. Biol. Chem. 204: 649–654. 1953.
19. Tolbert, N. E. and Zill, L. P. Metabolism of sedoheptulose-C^{14} in plant leaves. Arch. Biochem. Biophys. 50: 392–398. 1954.

20. VERNON, L. P. and ARONOFF, S. Metabolism of soybean leaves. II. Amino acids formed during short-term photosynthesis. Arch. Biochem. 29: 179–186. 1950.
21. WEISSBACH, A. and HORECKER, B. L. The formation of glycine from ribose-5-phosphate. A symposium on amino acid metabolism, W. D. McElroy and B. Glass, ed. Pp. 741–742. Johns Hopkins Press, Baltimore. 1955.
22. WITHROW, R. B., KLEIN, W. H., PRICE, L., and ELSTAD, V. Influence of visible and near infrared radiant energy on organ development and pigment synthesis in bean and corn. Plant Physiol. 28: 1–14. 1953.

PHOSPHORUS AND SULFUR COMPOUNDS IN PLANT XYLEM SAP[1]

N. E. TOLBERT AND H. WIEBE[2,3]
BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY,
OAK RIDGE, TENNESSEE

Since radioactive phosphorus and sulfur have become available, numerous investigators have determined the rate and conditions for the movement of these two essential materials from the soil through the roots to the leaves. The work has involved detection of the radioactivity as a measure of phosphorus or sulfur movement. It has been assumed that inorganic phosphate and sulfate were the major if not the only forms in which these elements moved in the upward-flowing xylem of the plant stem. Reports on phosphorus compounds in this plant sap are conflicting; Pierre and Pohlman (4) reported some phosphorus present as organic esters, while Lundegårdh (3) found no organic phosphates in the exudation of roots. The present investigation with radioactive tracers and paper chromatographic analyses indicates that, though inorganic phosphate is a major form of phosphorus being transported, there are in the xylem sap two other phosphorus-containing compounds one of which may account for a substantial portion of the transported phosphorus. Inorganic sulfate appears to be the only form of sulfur being transported.

PROCEDURES

Tests have been made with plants which produce a relatively large amount of exudate (bleeding) when their stems or leaf bases were cut off. Sacramento barley has been used in most of the experiments. The plants were grown in sand or soil in the greenhouse or in Hoagland's nutrient solution in a constant environment room with about 800 fc of light on a 16-hour day and at 75 to 77° F. In some experiments, the plants in sand or soil have been used as grown; in others, the sand or soil was carefully washed off the roots before placing them in 0.1 or 0.01 strength Hoagland's nutrient solution during the experiment to which P^{32} or S^{35} was added. Plants grown in sand or soil into which P^{32} phosphate was poured or injected yielded poorer results than those grown in aerated nutrient solution with P^{32}. The sand or soil bound the phosphate so tightly that the rate of appearance of P^{32} in the plant stem was more erratic. Flooding the sand or soil with the solution containing the tracer caused poor exudation. Polyethylene beakers were used for holding the P^{32} nutrient solution since the radioactive phosphate did not adhere tightly to the side wall of these containers. Plants were suspended by glass grids or by corks.

The radioactive phosphate or sulfate was placed in the dilute nutrient solution and at the same time or later the plant tops were removed near the base with a sharp razor blade. As the exudate appeared on the cut stump, it was removed completely by touching the origin point of a paper chromatogram to the stump. Thus for a given plant, the exudate could be transferred quantitatively, making it simple to carry out time-course studies. The first collections after cutting off the plant top utilized all the exudate that appeared. When collection times were further apart than ½ hour, there was often an excess of it which dripped from the stump or which was removed by blotting with a piece of filter paper. For these later collections the stump was blotted with filter paper ½ hour before the final sampling and all the exudate appearing after that was used for the chromatographic analysis.

Chromatograms were run on unwashed Whatman #1 paper. They were developed in the first direction with water-saturated phenol and in the second direction with butanol-propionic acid-water (1). After development, the chromatograms were exposed to no-screen x-ray film to establish the exact location of the P^{32}. These spots were cut into small pieces for counting with an end-window Geiger-Müller tube.

RESULTS AND DISCUSSION

On chromatograms of the exudate collected from the stump of decapitated barley seedlings, tomato, sunflower, bean, and willow there were 3 radioactive phosphorus spots which had R_f characteristics given in table I. The discovery of only 3 phosphorus-containing compounds in the exudate was surprising, since the phosphorus was neither all inorganic phosphate nor was there the multiplicity of known organophosphorus compounds associated with respiration which would be found in whole tissue. The two phosphorus-containing compounds besides inorganic phos-

[1] Received May 23, 1955.
[2] Work done while Dr. Wiebe was an Oak Ridge Institute of Nuclear Studies Research Participant at the University of Tennessee-Atomic Energy Commission Agricultural Research Program.
[3] Present address: Department of Botany and Plant Pathology, Utah State Agricultural College, Logan, Utah.

TABLE I

R_f OF UNKNOWN COMPOUNDS

COMPOUND	R_f	
	PHENOL-WATER	BUTANOL-PROPIONIC ACID-WATER
PO_4^{---}	0.25	0.25
Unknown (U_1) ...	0.9	0.3
Unknown (U_2) ...	0.35	0.2

phate have been designated U_1 and U_2. U_1 has the unusual R_f values of 0.9 in phenol-water and 0.3 in butanol-propionic acid-water, and it was labeled with much larger amounts of P^{32} than was U_2. The R_f value of 0.35 in phenol-water for U_2 is also high with respect to inorganic phosphate and the sugar phosphates of respiration. By their R_f values, the unknowns do not appear to be compounds usually associated with plant respiration; neither are they phospholipids which have large R_f values in the butanol-propionic acid-water solvent. In this paper are reported experiments to determine the general distribution and physiological variations of U_1 and U_2 in the exudate of decapitated plants. In addition, other investigations have shown them to be present in large amounts in both roots and leaves of higher plants.[4] Both results indicate that the unknowns may be important compounds in growth as well as in phosphorus transport.

COMPARISON OF XYLEM AND PHLOEM EXUDATE: The exudate from a decapitated stem or leaf base has generally been attributed to xylem flow from "root pressure." However, the xylem flow may not be solely responsible for the exudate, as phloem sap might also contribute to the bleeding. Consequently experiments were run with young willow plants, about 6 months after rooting, which could be barked to eliminate any phloem exudate. When this was done with P^{32} phosphate bathing the roots, U_1 and U_2 were found in the exudate regardless of whether the stem had been barked. Experiments were also run with branched willows from which exudate from each branch decapitated several inches above the fork could be chromatographed. In these latter experiments, one branch was barked and one was not, but in both cases U_1 and U_2 were present in the same portions in the exudate from the cut-off stems. These results are taken to indicate that U_1 and U_2 are constituents of the xylem sap.

To determine whether U_1 and U_2 might also be present in phloem sap, experiments were run with exudate from the squash peduncle and immature fruit. Undiluted radioactive P^{32} phosphate as received from the Radioisotope Department of Oak Ridge National Laboratory was placed directly on the leaves of a squash plant in the light with and without neutralization of the accompanying 0.02 *N* HCl. The P^{32} was transported rapidly throughout the plant as detected by a laboratory monitor. After 4 and 23 hours immature fruit was cut, both through the peduncle and through the fruit, and several collections of phloem exudate were made on chromatographic paper over extended periods of time. In all cases, no labeled U_1 nor U_2 appeared on the chromatograms. The P^{32} was present primarily as inorganic phosphate. The negative results for the presence of U_1 and U_2 indicate that these unknown phosphate esters were not constituents of the squash peduncle exudate which is considered phloem sap (2).

PERCENTAGE P^{32} IN UNKNOWN PHOSPHATE ESTERS: Distribution of the radiophosphorus between the 3 compounds in the xylem exudate of decapitated plants showed consistent physiological variations. Table II shows that the percentage of the total P^{32} in the unknowns in tomato stem exudate increased with the time after applying the radioactive phosphate to the nutrient solution surrounding the roots. In these experiments, the plant top was cut off at the same time the P^{32} was supplied to the nutrient solution. The Pan-American tomato plants were approximately 45 days old and had been grown in soil to which the P^{32} was supplied without disturbing the roots. The percentage of P^{32} activity in U_1 was about 20 % of the total in the exudate 6 hours after decapitating the plant, and U_2 remained present in very small amounts. Since all the P^{32} was in these compounds, the decrease in percentage P^{32} in inorganic phosphate was almost completely reflected in the rise of P^{32} in U_1.

With Sacramento barley (table III) the percentage of P^{32} in U_1 was less than for tomato but the actual amount of P^{32} in U_1 and U_2 was greater; it exceeded by far that necessary for chromatography and counting purposes. Consequently, barley seedlings were used for further investigations because of their rapidity of growth and ease of handling. In many different experiments with older green Sacra-

TABLE II

DISTRIBUTION OF P^{32} IN UNKNOWNS OF EXUDATE FROM TOMATO STEMS*

TIME**	TOTAL P^{32} †	PERCENTAGE ACTIVITY		
		PO_4^{---}	U_1	U_2
hr	*c/s*			
½	9.7	100.0	Trace	0.0
1	70.9	98.4	1.6	Trace
1½	154.9	97.2	2.8	Trace
2	193.2	95.0	4.8	0.2
3	302.7	90.3	9.3	0.4
4	207.1	87.4	12.3	0.3
6	229.5	79.4	20.3	0.3

* Pan-American tomatoes, 45 days old.

** Time from simultaneous application of P^{32} to the roots and cutting off the stem.

† Values represent counts per second (c/s) on the chromatogram from approximately ½-hour collection periods just prior to the time record in the first column.

[4] TOLBERT, N. E. An unknown phosphate ester abundant in plants. In: General Program, Amer. Assoc. Advance. Sci., Sect. FG 7, Soc. Gen. Physiol. P. 234. Ann. meeting, Berkeley, California, 1954.

TABLE III

RATE OF P^{32} APPEARANCE IN U_1 AND U_2 OF THE EXUDATE FROM DECAPITATED BARLEY PLANTS

TIME AFTER P^{32} SUPPLY (HR)	PERCENTAGE OF U_1*		PERCENTAGE OF U_2*	
	t = 0 **	t = 4 ***	t = 0 **	t = 4 ***
¼	0.0	....	0.0	....
½	1.22	....	0.20	....
1	2.67	....	0.22	....
2	1.50	....	0.27	....
4	4.91	....	0.77	....
4¼	....	4.69	....	0.91
4½	....	5.06	....	0.93
5	4.91	4.47	0.69	0.76
6	4.59	3.47	0.62	0.87
8	4.88	6.30	0.61	1.05

* Percent of total P^{32} of the exudate in the unknowns as analyzed by paper chromatography. The remainder was in inorganic phosphate.

** For plants which were decapitated at zero time of column one or at the same time the P^{32} phosphate was added to the nutrient solution.

*** For plants which were decapitated 4 hrs after adding the P^{32} phosphate.

mento barley plants, 3 to 5 % of the P^{32} in the exudate was found in U_1. The amount of P^{32} in U_2 paralleled U_1 and usually amounted to approximately 1/10 the activity of U_1.

With stems of Black Valentine bean plants, sunflower, and willow, it was more difficult to obtain exudate, but fresh cuts on the stem could be made over a period of several days. The percentage of the total P^{32} in U_1 and U_2 was much lower than that for the tomato plant and was of the order of magnitude of that for barley. As before, only three compounds in the exudate were labeled with the P^{32}, namely inorganic phosphorus and U_1 and U_2.

PRESENCE OF U_1 IN GUTTATE: In the guttation fluid from the tips of young barley plants, U_1 can be easily detected a few hours after P^{32} phosphate has been applied to the roots. The amount of U_1 in the guttate is very low compared to that in the xylem exudate. U_2 has not been detected in the guttate. Because of the very small amount of U_1 in the guttate, only traces of U_2 would be present if the two substances remained in the ratio found in the xylem exudate. The major P^{32}-containing substance in the guttate was inorganic phosphate. The presence of small amounts of U_1 in the guttate is another indication that U_1 is a constituent of the xylem sap in the intact plant.

RATE OF INCORPORATION OF P^{32} INTO U_1: For Sacramento barley seedlings, the appearance of P^{32} in U_1 and U_2 of the exudate reached a constant, maximum percentage value about 4 hours after the addition of P^{32} to the nutrient solution bathing the roots. This time lag of 4 hours was independent of when the plant top was cut off for collection of exudate. In table III is given the percentage of P^{32} in U_1 and U_2 in the exudate when the leaf was decapitated at the same time the P^{32} was added to the nutrient solution (t = 0) or 4 hours later (t = 4). In both cases, the amount of P^{32} in U_1 and U_2 were the same after 4 hours within the wide physiological variations. The data imply that the incorporation of P^{32} phosphate into U_1 and U_2 was at a rate limiting speed which was much slower than the transport of P^{32}-labeled inorganic phosphate in the xylem flow. The absence of any labeled U_1 in the exudate during the first 15 minutes after putting P^{32} phosphate into the nutrient medium and the slow increase over 4 hours in the percentage of P^{32} in U_1 was not caused by a lack of P^{32} inorganic phosphate in the root or in the xylem exudate. Rather, this period of about 4 hours for barley seedlings before U_1 reaches maximum P^{32} activity was more likely a measurement of the rate of incorporation of inorganic phosphate into U_1 and U_2.

POSSIBILITIES THAT U_1 AND U_2 WERE ARTIFACTS OF THE EXPERIMENT: Several experiments all indicate that U_1 and U_2 were normal constituents of the xylem exudate rather than wound compounds on the cut surface of the stem or leaf or products formed by radiation injury from the P^{32}.

(a) The time lapse described above in appearance of labeled U_1 and U_2 after P^{32} phosphate appeared in the exudate indicates that these substances were not wound produced. If they had been abnormal wound compounds the percentage of P^{32} in them should have been the same at equal time intervals from decapitating the plant. This was not the case, but rather the unknowns appeared immediately on the cut surface only if at least 4 hours had elapsed after adding the P^{32} before decapitating, which time was necessary to equilibrate the pools of the unknowns normally present with the added P^{32}.

(b) The discovery of large amounts of these two substances in the roots and leaves of barley indicated that they are normal constituents of the plant (5).

(c) Since the percentage of P^{32} in U_1 lagged behind the appearance of P^{32} phosphate in the xylem exudate, U_1 might possibly be a product of radiation damage from the P^{32}. Consequently young barley seedlings were placed in solutions containing the same total phosphate concentration but a ten thousand fold range in specific activity of the P^{32}. Exudate samples were chromatographed from decapitated seedlings at each concentration used, and the total P^{32} and the percentage of it in U_1 are shown in table IV. The percentages of P^{32} in U_1 and U_2 were the same in all cases and were entirely independent of the specific activities of P^{32} phosphate in the nutrient. Thus the radioactivity from the P^{32} in the tracer concentrations used in these short experiments had no significant effect on the amounts of the unknowns present.

(d) The possibility that the unknowns might have been formed by the cells on the surface of the cut tissue and then excreted into the exudate was excluded by two series of experiments. 1) Tomato stems and barley leaf bases were cut off and 4 hours afterwards neutralized P^{32} phosphate was added by dropper to the cut-off surface. Still later, the exudate on the stem was chromatographed. No U_1 or U_2 was

TABLE IV

EFFECT OF THE SPECIFIC ACTIVITY OF PHOSPHATE ON PERCENTAGE DISTRIBUTION OF P^{32} IN U_1 OF BARLEY EXUDATE

SPECIFIC ACTIVITY *	TOTAL P^{32} IN COLLECTION **	AMOUNT OF U_1
μc/l	*cpm*	*% total P^{32}*
0.002	17	~ 15.5
0.02	157	8.2
0.2	3,230	8.8
2.0	24,500	9.1
20.0	156,000	9.0

* Total phosphate concentration was held constant at 10^{-3} M. Otherwise, nutrient solution was 0.01 Hoagland.
** Exudate collected 25 hrs after adding P^{32} phosphate and cutting off the leaves. Data expressed as P^{32} collected per plant. Similar results were obtained at other collection times.

found in these exudate from the cut-off tomato plants nor from the barley plants in the time intervals which were being considered. After a time lapse of hours P^{32} label did appear in U_1 and U_2 from the barley plants which might be attributed to transport of the P^{32} to the roots and its reappearance in xylem exudate. 2) Ten tomato stem slices or barley leaf slices (20 times the surface exposed on a decapitated stem) were incubated in about 2 ml of 0.01 *N* sodium acetate solution containing approximately 1 μc of P^{32} inorganic phosphate. Small aliquots of the supernatant were removed at 2, 4, 6, 24 and 60 hours and chromatographed. From barley leaf slices, only trace amounts of labeled phosphate esters appeared in the supernatant until after 60 hours, while P^{32} labeled compounds were excreted by the tomato stem slices after 6 hours of incubation. The excreted P^{32} labeled substances included not only U_1, but also large amounts of phospholipids and phosphate compounds with R_f values of sugar phosphates. This rather indiscriminate loss of compounds would appear to reflect cell destruction and death in the tissue slices. Only a small amount or none of U_1 and U_2 selectively appearing immediately in the xylem exudate when the plants were decapitated can be explained on the basis of excretion from the exposed cells on the cut surface.

(e) The possibility that the unknowns might have been formed in the exudate by extra cellular enzymes was excluded by collecting large quantities of exudate from barley seedlings and placing it in a test tube with neutralized P^{32} phosphate. Exudate collections were made ½, 1, 2, 4, 6 and 24 hours after cutting the stems, and the exudate was allowed to stand at room temperatures in the P^{32} phosphate solution for 2 hours before being chromatographed. No P^{32} containing spots other than inorganic phosphate appeared on the chromatograms from any tomato exudates or from barley exudate of the early collections. In barley exudate collected 24 hours after cutting off the stem, a small amount of P^{32} labeled U_1 was present after incubation with the added P^{32} phosphate.

EFFECT OF AGE OF BARLEY SEEDLINGS: An important physiological factor affecting the amount of U_1 and U_2 in the xylem exudate of cut-off barley leaves was the age of the plant. Experiments concerning this factor have been done with barley seedlings grown either in Hoagland's nutrient solution or in sand supplied with nutrient solution. Sand was carefully washed off the roots and these plants were placed in beakers of aerated 0.01 strength Hoagland's nutrient solution. The tops were decapitated simultaneously with addition of P^{32} to the nutrient solution, and exudate samples were collected for 24 hours. In tables V and VI are reported the percentages of the P^{32} in the exudate present in U_1 and U_2 for barley plants grown in nutrient solution and varying in age from 3 to 28 days. In addition, in order to show the amount of P^{32} in the unknowns, there is given the total counts per second of P^{32} in either U_1 or U_2 that was collected on each chromatogram from each age of plant. These values do not represent the total P^{32} exuded, for much of the exudate ran to waste, but rather the amount acquired in the collection period, which was consistent for each group of plants. Similar data were obtained for collection period 2, 6, 16, 22, and 28 hours after the beginning of the experiment.

The data indicate that the percentages of P^{32} in U_1 or U_2, as well as the amount of P^{32}, varied with age of the seedling. There was a much higher percentage and amount of the P^{32} from the nutrient solution in U_1 and U_2 in the youngest seedlings, especially the 3- and 4-day-old seedlings. With increasing age of the seedling, the amount and percentage of P^{32} in U_1 and U_2 decreased markedly to minimum values at a plant age of about 9 days. This minimum varied somewhat, probably depending on the rate of metabolism of the seedling, and corresponded with the time when, by visual observation, the seed shriveled up

TABLE V

EFFECT OF AGE OF BARLEY SEEDLING ON AMOUNT OF P^{32} IN U_1*

AGE OF SEEDLING	COLLECTION TIME, HRS							
	4		8		12		24	
days	*c/s*	*%*	*c/s*	*%*	*c/s*	*%*	*c/s*	*%*
3	4.3	3.1	11.5	3.2	36.1	2.8	3.5	2.4
4	8.6	1.8	10.6	2.5	3.6	1.6	2.5	1.8
6	0.4	1.0	7.4	1.7	...	...	4.5	1.5
9	Trace	Trace	1.8	0.6	Trace	Trace	1.4	0.6
12	0.8	0.3	1.8	0.4	1.8	0.1	5.3	1.3
16	3.2	0.2	11.1	0.6	2.8	0.5	16.0	0.9
22	1.8	0.3	15.8	0.4	5.4	0.5	14.0	1.2
28	0.8	0.3	10.9	0.5	13.2	0.5	43.0	2.2

* Values are not corrected for radioactive decay, but all samples were counted on the same day. The figures are those counted from the chromatogram after preparation of the radioautograph to locate the radioactivity and were several-fold larger at the beginning of the experiment. In calculating the percentage of P^{32} a correction for radioactive decay was made back to the time of counting U_1 for the radioactivity in inorganic phosphate which was counted at a later date.

TABLE VI

EFFECT OF AGE OF BARLEY SEEDLING ON AMOUNT OF P^{32} IN U_2

AGE OF SEEDLING	COLLECTION TIME, HRS							
	4		8		12		24	
days	*c/s*	*%*	*c/s*	*%*	*c/s*	*%*	*c/s*	*%*
3	0.4	0.3	1.4	0.4	4.7	0.4	0.7	0.5
4	1.6	0.3	0.8	0.2	0.9	0.4	0.7	0.5
6	0.1	0.2	1.8	0.4	...	...	0.7	0.2
9	~ 0.0	~ 0.0	0.7	0.2	~ 0.0	~ 0.0	0.4	0.2
12	< 0.1	< 0.05	1.0	0.2	0.3	< 0.05	0.4	0.1
16	0.7	< 0.05	1.5	0.1	0.1	< 0.05	1.7	0.1
22	0.5	< 0.1	4.3	0.1	1.1	0.1	2.0	0.2
28	< 0.1	< 0.1	3.3	0.2	4.1	0.2	10.8	0.6

and was apparently exhausted. At this time, the total exudate and total P^{32} in the exudate were at a minimum. With further increase in age of the plant, the amount and percentage of P^{32} in U_1 and U_2 again increased, and with the older plants grown in sand or soil the percentage of P^{32} in the unknowns reached a higher level.

These results suggest that in the young plant the seed was contributing to the synthesis of the two unknowns with the P^{32} phosphate absorbed from the nutrient solution. As the seedling aged, a period of exhaustion occurred after which, the roots appeared to assume the entire function of production of U_1 and U_2. Certainly, this must have been true for the tomato plants that were used at 1 to 2 months of age. For them, no seed remained although large amounts of U_1 were present in the exudate.

It has been assumed that all of the phosphorus in the exudate has been furnished by the nutrient solution to allow recording the data as percentages of the total radioactivity in the exudate. This assumption might result in values too low for the amount of U_1 and U_2 from the youngest seedlings since the phosphate in U_1 may be partially supplied by the seed which contained no P^{32}. During early stages of germination, the complete formation of the unknowns from material including phosphorus of the seed might be expected. However, no more satisfactory way to express the data has been found than as percentage of total P^{32} in the exudate.

In these experiments on the effect of age on the amount of U_1 and U_2, the use of nutrient solution to grow the older plants was not so satisfactory as sand culturing. Although the older plants in nutrient solution appeared almost as healthy as sand grown plants, the capacity of the roots from the older plants to supply U_1 and U_2 was not nearly so great. This was probably related to our inability to raise barley seedlings to maturity in nutrient solution.

EFFECT OF REMOVING THE SEED FROM YOUNG PLANTS: The hypothesis arrived at by studying the amount of U_1 and U_2 in exudate from seedlings of varying age was that the seed makes a major contribution to the formation of these esters. This was verified by experiments in which seeds were removed from young barley seedlings in aerated nutrient solution before, at the same time, or after addition of the P^{32} phosphate and simultaneously with decapitation of the leaf. In figure 1 is plotted the rate at which labeled P^{32} appeared in the exudate from 4-day-old barley seedlings. In the control experiment, in which the seed was left on the seedling, the percentage of the total P^{32} in U_1 reached a maximum value of about 3 % around 5 to 6 hours after addition of the P^{32}. If the seed was removed either at the beginning of the experiment or 24 hours earlier, the amount of P^{32} in U_1 remained at a constant low value of 0.6 to 0.9 % of the total P^{32} of the exudate. This level is presumably the amount which the roots were able to supply at this age of the barley seedlings. When the seed was left on for the first 4 hours of the experiment to permit a buildup toward the maximum amount of P^{32} in U_1, and then removed, U_1 decreased markedly after 2 hours. In these latter seedlings, there was no decrease in the amount of P^{32} in U_1 1 hour after the seed was removed but there was not the continued increase as there was in their controls. Apparently, removal of the seed did not affect the xylem transport mechanism, and the loss of the seed was reflected only in the lowered production of U_1. Within 2 hours, the amount of P^{32} in U_1 was dropping owing to its exhaustion in the remaining, lower part of the plant. This drop in amount of U_1 was to a level about equal to that in plants from which the seed had been removed at the same time P^{32} was supplied to the roots. The results suggest that the seed was contributing directly to the synthesis of the unknown phosphate compound. Until the compound is identified, it cannot be determined whether the seed was furnishing only energy or organic components as well.

SULFUR TRANSPORT: Radioactive S^{35} inorganic sulfate has also been used with barley plants in experiments similar to those described for detection of the

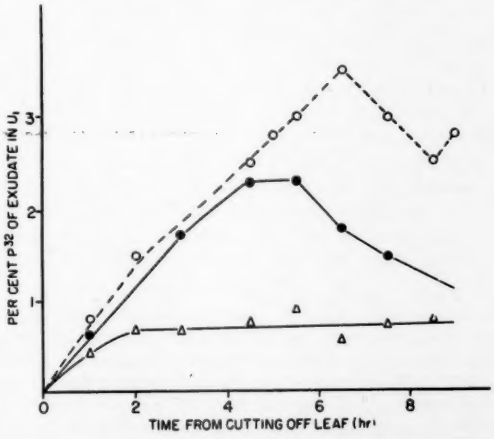

FIG. 1. U_1 in exudate from 4-day-old barley seedlings. ○, seed not removed; ●, seed removed at 4th hr of experiment; △, seed removed at beginning of experiment.

P^{32} labeled compounds of the xylem exudate. Only radioactive inorganic sulfate was present on chromatograms of the exudate collected over 24 hours. The S^{35} inorganic sulfate in the exudate fluid was isolated chromatographically as easily as the phosphate, but no detailed study of time versus amount of S^{35} in the exudate has been run.

Summary

The xylem exudate of several species of plants contained three P^{32} labeled compounds—inorganic phosphate, and two unknown phosphorus containing compounds. As much as 20 % of the total radioactive phosphorus has been observed in the unknown compounds from the exudate on decapitated tomato stems. Sulfur was present only as inorganic sulfate.

The two unknown phosphorus-containing compounds are normal constituents of the xylem sap, but they have not been detected in the exudate from squash peduncle. The unknown present in largest amounts in the exudate from decapitated plants was also found in barley guttate. The two unknowns are normal constituents of the xylem exudate rather than wound compounds on the cut surface of the stem or leaf base or products formed by radiation injury from the P^{32}.

The percentages of P^{32} in the unknowns reached a maximum value about 4 hours after the addition of P^{32} to the nutrient solution bathing the roots. This time lag was independent of when the plant was decapitated to collect exudate. It is interpreted as indicative of the rate of incorporation of phosphate into these compounds.

Both the seeds of seedlings and the roots of older plants contributed to the formation of the two unknown phosphorus-containing substances. Removal of the seed from young seedlings severely reduced the amount of these compounds present. The amount of the unknowns in the xylem exudate decreased rapidly with age of the barley seedling, but remained present in considerable quantity in older plants.

Literature Cited

1. Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. and Stepka, W. The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. Jour. Amer. Chem. Soc. 72: 1710–1718. 1952.
2. Crafts, A. S. Further studies on exudation in cucurbits. Plant Physiol. 11: 63–79. 1936.
3. Lundegårdh, H. Absorption, transport and exudation of inorganic ions by roots. Arkiv Bot. 32A, no. 12: 1–139. 1945.
4. Pierre, W. H. and Pohlman, G. G. Preliminary studies of the exuded plant sap and the relation between the composition of sap and the soil solution. Jour. Amer. Soc. Agron. 25: 144–160. 1933.

TRANSIENT CHANGES IN CELLULAR GAS EXCHANGE AND THE PROBLEM OF MAXIMUM EFFICIENCY OF PHOTOSYNTHESIS[1,2]

ROBERT EMERSON and RUTH CHALMERS

Botany Department, University of Illinois, Urbana, Illinois

I. Formulation of Issues

Since 1938 different investigators have reached very divergent conclusions regarding the maximum efficiency of photosynthesis, in spite of the fact that most of the calculations of efficiency have been made from measurements of the rate of photosynthesis of the green alga, Chlorella. For example, Rieke (25), Emerson and Lewis (11), and Daniels with various co-workers (15) found that the efficiency of photosynthesis did not exceed a value of about 30 %, while Warburg (30) with various co-workers (6, 7, 31, 34) has published a series of papers in which he reports efficiencies of 70 % and upwards to 100 %. We refer here to measurements in the red region of the spectrum where the carotenoid pigments do not contribute to light absorption. This region, near the long-wave limit of light absorption by chlorophyll, should favor the attainment of maximum possible efficiency, if the quantum yield is independent of wave length.

From published discussions of the subject, it seems clear that the great differences in calculated efficiency are due primarily to differences in the estimation of the rate of photosynthesis, rather than to disagreement as to the absorbed light energy. Questions have arisen concerning the application of certain specialized techniques for measuring the rate of photosynthesis. Most of the rates from which efficiencies of 70 % and higher have been calculated, are derived from manometric measurements of pressure changes in the gas space above aqueous suspensions of cells. In general these measurements have been made with cell suspensions dense enough to absorb practically all the energy of the incident beam of measured light.

There are some reports (Warburg, 30; Damaschke et al, 8) of confirmatory results from measurements with thin suspensions of cells, by both manometric and electrometric methods. We shall not discuss these

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measurements in the present communication, because they raise issues different from those connected with the measurements with dense suspensions and short periods of exposure to measured radiation. Since these latter measurements constitute the principal foundation for the claims of very high efficiencies, it seems appropriate to give them primary consideration.

Disagreement concerning the significance of manometric measurements with dense suspensions of cells stems from disagreement as to the proper allowance for time delay or lag between changes in rate of metabolic gas exchange inside the cells, and the attainment of corresponding rate changes in the observable motion of the manometric fluid, from which the rate of photosynthesis is calculated. Changes in rate are necessarily involved, because the rate of pressure change attributable to photosynthesis is not directly observable but must be derived from the difference between two observed rates, one when the cells are exposed to a light beam of measured intensity, and the other when the cells are darkened. In principle it is of no consequence whether the cells are darkened between exposures to the measured light, or whether the measured beam is superimposed from time to time upon a background of continuous illumination from some other source (a practice often followed by Warburg and co-workers in their measurements of efficiency). In either case the rate of pressure change attributable to the beam of measured light must be derived from the difference between two observed rates, so there must be a change from one rate to another between the two observations.

If rates of photosynthesis are calculated from rates of pressure change observed in close succession after changes in illumination, neglect of the effects of time lag can lead to error in the calculated rates of photosynthesis, and hence to error in the estimation of efficiency.

Warburg and co-workers have expressed the opinion that in their application of the manometric technique, time lag between changes in rate was of negligible importance. Their published values for the efficiency of photosynthesis are derived from rates of pressure change in close succession after changes in illumination, at intervals sometimes as short as one minute (34), without any allowance whatever for time lag.

Others who use the manometric technique for studying photosynthesis have observed time lags of several minutes between changes in illumination and the approximate attainment of new steady rates of pressure change (19). The apparent absence of time lag reported by Warburg and co-workers therefore calls for explanation. One possibility is that the frequency and amplitude of shaking which they specify are sufficient to make the approach to equilibration between gas and fluid space so rapid that time lag is, as they suppose, of negligible importance. An alternative explanation is that the time lag in their manometer system is much the same as is commonly observed in other laboratories, but that under the conditions of their experiments, compensatory processes obscure the effects of time lag. Our first objective is to present experimental evidence showing that under conditions closely approximating those specified by Warburg and co-workers, time lag is appreciable, and follows a course which would be expected if it were due primarily to diffusion between liquid and gas space. Absence of apparent evidence of time lag is therefore to be regarded as evidence of compensatory processes, and should not be interpreted as indicating that the manometric pressure changes respond without time lag to changes in metabolic rates.

This makes it necessary to consider the nature of the errors which would be introduced by neglecting the effects of time lag due to diffusion. In the case of measurements of rate of exchange of a single gas, calculated from pressure changes in a single manometer vessel, the neglect of diffusion lag would ordinarily result in underestimation of the magnitude of any change in rate, and hence in underestimation of the rate of photosynthesis. Roughton (26) has shown that by application of a simple correction for the effect of diffusion, it is possible to calculate the actual rate of gas production, even during the lag period. But Warburg and co-workers have made their measurements by the so-called two-vessel method, in order to follow the rates of production or consumption of two gases, oxygen and carbon dioxide. In the case of two-vessel measurements, the effects of diffusion lag are more complex. The technique calls for the measurement of two rates of pressure change, in two reaction vessels containing equal samples of cell suspension. It remains true that the neglect of time lag will generally result in underestimation of the magnitude of changes in rates of pressure change in each reaction vessel, but in the two-vessel measurements the rates of oxygen and carbon dioxide production are functions of the ratio of the rates of pressure change in the two vessels, so the effect of diffusion lag upon calculated rates of gas production will depend upon its effect on this ratio. (We shall use the term "production" to cover both production and consumption, since consumption may be regarded as negative production.) The application of the two-vessel method presupposes not only that the metabolic rates of gas production remain equal in the two vessels at all times, but also that during periods of time lag the rates of diffusion of gas between liquid and gas phase remain equal in the two vessels. These requirements must be fulfilled with respect to both oxygen and carbon dioxide. If diffusion lag is not equal in the two vessels, then neglect of its effects could result in either under- or overestimation of the rates of gas production.

The problem will be further complicated if at the times of change in illumination the rate of metabolic gas production does not come immediately to its new value, but undergoes some transient changes before it reaches a steady value. This will lengthen the period during which errors may arise from differences in diffusion lag in the two vessels.

We have mentioned above that we shall present evidence (Part III) which we interpret as indicating

that there are compensatory processes which can obscure the effects of diffusion lag. These compensatory processes bring about transient changes in rates of gas production. We shall also present evidence (Part IV) that in vessel pairs like those used by Warburg and co-workers there are sometimes substantial differences in diffusion lag. Therefore the contingencies discussed in the preceding paragraphs may actually be encountered, and are not mere hypothetical possibilities.

In their publications, Warburg and co-workers have not dealt with these contingencies. Doubtless they thought it unnecessary because in their experiments they noticed no evidence of time lag. But our evidence that time lag is nevertheless a significant factor under the conditions of their measurements leads inevitably to the conclusion that the next step toward assessing the significance of their measurements of efficiency of photosynthesis is the comparison of diffusion lag in vessel pairs such as they describe. We shall present measurements which indicate that equality of diffusion lag cannot be assumed for such vessel pairs, but that by modifying the shape of one of the members of the pair it is possible to obtain pairs which show equality of diffusion lag with respect to one gas (oxygen) within the limits of sensitivity of the method. The establishment of equality of diffusion lag with respect to one gas is an improvement over the uncertainty which prevails in the case of the measurements of Warburg and co-workers, where it is probable that diffusion lag was unequal with respect to both gases. However, equality of diffusion lag with respect to one gas implies, as we shall explain in Part IV, inequality with respect to the other. This also must be taken into consideration in the application of the method.

It is appropriate to mention that Warburg et al (35) have recently noticed the effects of time lag in manometric measurements of photosynthesis, and have reported making allowances for these effects. They report no change in their conclusions concerning the efficiency of photosynthesis. The reader might infer from this that our worries about possible errors from time lag in earlier experiments are groundless, and that since Warburg has now begun to make allowance for the effects of time lag, the work which we report here might now be regarded as superfluous. To any reader who may be inclined to adopt this viewpoint, we would reply that the experiments in which no allowance was made for time lag remain the principal foundation of Warburg's fundamental claims regarding the efficiency of photosynthesis. Warburg and co-workers have yet to publish any measurements of time lag which would provide a basis for deciding whether the allowance they now propose to make for it is adequate. They have yet to recognize the tendency of transient metabolic processes to prolong the period of diffusion lag; and their two-vessel measurements are still made with vessel pairs for which there are no published data on equality of diffusion lag. Under these circumstances, discussion of the issues we have raised in this communication is not superfluous.

II. Experimental Methods

Since our ultimate purpose is to assess the significance of Warburg's conclusions derived from two-vessel measurements of the rate of photosynthesis, we have followed his specifications in many respects. However, in order to obtain greater precision of measurements of pressure change, it has also been necessary to introduce some changes.

We have followed the recent practice of Warburg and co-workers, and used rectangular reaction vessels shaken with a horizontal linear motion, instead of the larger circular vessels shaken with rotary motion which had been used by Warburg and Negelein (36) and also by Emerson and Lewis (10, 11). The combination of rectangular vessel shape and horizontal shaking leads to less foaming of the cell suspension than the rotary shaking in circular vessels, and also makes it easier to shake two vessels simultaneously with identical motion.

Warburg's two-vessel measurements of photosynthesis have been made with the open, constant-volume type of manometer. Three are required for an experiment—two for the two samples of cell suspension, and a third to correct for changes in barometric pressure. Two visual estimations of fluid level enter into the reading of each manometer; one for the pinch-cock adjustment to constant volume, and one for the pressure reading itself. Since the barometric pressure correction contributes to each measurement of metabolic pressure change, four visual estimations of fluid level enter into each measurement. All these estimations are made while the manometers are in rapid motion. Even with the aid of a hand lens, a precision of $\pm$ 0.5 mm is the utmost that can be expected. If the pressure changes are 5 mm or less, the random errors of $\pm$ 0.5 mm are important. In the experiments of Warburg and co-workers, many rates of photosynthesis are derived from pressure changes of about 3 mm. A change read as 3.0 mm might be anything from 2.5 to 3.5 mm. Here the range of uncertainty is 30 %. Nishimura et al (20) have given examples of the errors that can arise in the computation of rates from two-vessel measurements, through reading errors which would be of only minor importance in single-vessel measurements. Pirson et al (24) have given a more general and extensive discussion of random errors in the two-vessel method, from which it may be seen that an error of 30 % in one of the two rates of pressure change can lead to an error of several fold in the rate of gas production.

Greater precision is attainable by reading the manometers with a cathetometer (horizontal telemicroscope with cross-hairs and screw adjustment for height, and scale divided into hundredths of a mm), but the constant-volume type of manometer does not lend itself to reading by cathetometer because a barometric pressure correction must be read in addition to the pressure changes for the two cell samples, and because of the pinchcock adjustment for constant volume. In order to avail ourselves of the greater precision of the

cathetometer, we have substituted differential manometers for the constant-volume type used by Warburg. The differential manometer is a closed system, and requires no control for barometric pressure changes nor adjustment to constant volume. For two-vessel measurements only two manometers are required, each with a compensation vessel and a vessel with cell suspension. One manometer with its two vessels is illustrated in figure 1.

The manometers were of uniform-bore pyrex capillary with an area of cross section of 0.426 mm^2.

One might suppose that a double cathetometer unit (two telemicroscopes) would suffice for reading two differential manometers. In the case of each manometer, if the fluid level rises on one side it should fall an equal amount on the other, so a single reading should suffice to establish the change in pressure. However,

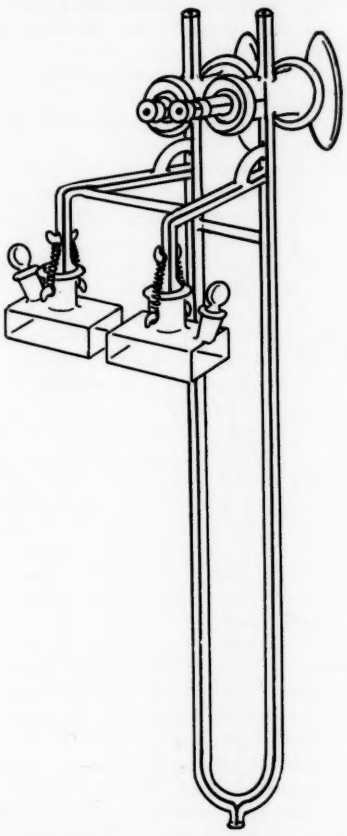

FIG. 1. Differential manometer, with vessels of the shape used for measuring H. One vessel is used as a compensating vessel, with suspending fluid but no cells. The other vessel is for the experimental material. For two-vessel measurements, a second manometer is required, with a pair of vessels larger than the pair shown here. The shapes of the two types of larger vessels used are shown in figure 2 (shapes B and C).

FIG. 2. Vessel shapes used for two-vessel measurements. Vessels of shape A were used for measuring H. For measuring h, Warburg and co-workers used vessels of B shape (like A except deeper). We have used vessels of C shape for measuring h. The depth is the same as for the A vessels, except for the central tower.

the ascending meniscus does not always travel the same distance as the descending one, because on the descending side manometer fluid is left on the wall of the capillary tube, while on the ascending side the meniscus may be moving up into dry tubing, or may be adding to its level by picking up fluid left on the wall during a previous descent. The simultaneous reading of both meniscus levels minimizes (but does not eliminate) errors arising from the wetting of the capillary walls by the manometer fluid. A quadruple unit is therefore necessary for reading two differential manometers. The simultaneous setting of all four columns by four observers presented too many difficulties, and we found that with practice, two observers could set all four columns within four seconds, and repeat the process at intervals of one minute, for many minutes. The four-second interval between settings introduces no error when the rates of pressure change are constant. There is, of course, a small error when the rates change between one set of readings and the next. We have not attempted to make a correction for this error, which is small compared with random errors. The four cathetometer settings within four seconds are more nearly simultaneous than naked-eye readings of three constant-volume type manometers, an operation which often takes 20 seconds.

Although the cathetometers are read to the nearest hundredth of a mm, the precision is not as great as this. Besides the errors introduced by wetting of the capillary walls, there are errors due to back-lash in the cathetometer screws, and the presence of the observers causes noticeable effects on the apparent meniscus levels, probably through warming of the metal parts of the cathetometer. Systematic attention to minor details does not seem to improve the precision beyond about ± 0.03 mm. However, this represents an improvement of more than ten-fold in the precision attainable with the constant-volume manometers.

We have compared pairs of reaction vessels similar to the ones described by Warburg and co-workers and also other patterns. Figure 2 shows the three shapes with which we have done most of our work. Shapes A and B represent the pair described by Warburg and Burk. These two vessels are supposed to differ in depth only. We have found that all hand-formed rectangular vessels of this type differed in other dimensions as well as depth. In agreement with Nishimura

et al (20, pp. 200–201, table 5) we always found significant differences in diffusion lag in such vessel pairs, but, because of the differences in other dimensions as well as depth, it was not possible to be sure whether the observed differences in lag were characteristic of differences in depth. Recently we have obtained (from Fischer and Porter Co.) vessels made to within 0.1 mm of specified dimensions, from pre-formed rectangular pyrex tubing. With such vessels we have been able to compare diffusion lag in pairs which differed in depth alone, and these tests, described in Part IV, have been the basis of our conclusions regarding the comparability of diffusion lag in vessel pairs of shapes A and B.

The shape C shown in figure 2 represents the kind of vessel suggested by Nishimura et al (20, pp. 189, 206), as a substitute for Warburg's deep vessel. The space in which the cell suspension circulates is supposed to be the same as in the A shape, the required difference in volume being obtained by adding the central tower, instead of increasing the depth over the entire area. Nishimura et al (20) thought that with an A-C pair differences in diffusion lag would be less likely than with an A-B pair.

We are now able to obtain C-shape vessels made to accurate dimensions from rectangular tubing. However, all experiments with C-shape vessels reported in this paper were done with hand-formed vessels, matched with hand-formed vessels of the A shape, since precision-formed vessels of the C pattern did not become available until the work was nearly completed.

Since our purpose was to study the effects of diffusion lag under conditions approximating those prevailing in the measurements of Warburg and coworkers, we adjusted conditions to be at least as favorable to diffusion of gas between liquid and gas phase as in their experiments. Diffusion rate is a function of the area of liquid surface, the liquid volume, the irregularities in the internal shape of the vessels, and the amplitude and frequency of the shaking motion. Warburg et al (33, pp. 337–338) describe their vessels as about 22×38 mm in internal width and length, and having volumes of either 13 to 14 ml or 18 to 19 ml, depending upon depth. In most of the experiments which they report with such vessels, they used 7 ml of cell suspension. Our hand-formed vessels of shapes A and C were of comparable length and width. Their volumes were 14.9 or 18.4 ml, depending on whether the central tower was present. In such vessels we also used 7 ml of suspension.

For our tests of equality of diffusion lag in vessel pairs like the A-B combination, we used the vessels made from precision-formed rectangular tubing, accurate to 0.1 mm in all internal dimensions, in order to exclude the effects of all differences except depth. These vessels were 26×40 mm in internal width and length, thus providing a considerably larger surface area than the vessels described by Warburg and coworkers. The volumes were 14.7 ml or 22.1 ml, depending on whether the internal depth was 13 or 20 mm. In such vessels we used either 7 or 8 ml of suspension. The 8 ml filling still gives a more favorable surface-volume ratio than the 7 ml filling in a vessel 22×38 mm (dimensions specified by Warburg), so that with the 8 ml filling our vessels should still provide more favorable diffusion conditions than his.

In different publications Warburg and co-workers have specified shaking amplitudes of from 1.7 to 2 cm and frequencies of from 140 to 200 per min (for example, Warburg et al, 33, p. 337, 140–180 per min; Burk and Warburg, 7, p. 14, 200 per min). Throughout our work we have used amplitudes of 1.8 cm and a frequency of 200. Higher speeds or larger amplitudes result in occasional splashing of the liquid into the entrance of the capillary tube connecting manometer with reaction vessel, with consequent interruption in the sequence of readings.

From 200 to 300 μl of cells were used in each vessel. This amount of cells gives total absorption of a beam of red light ($\lambda = 644$ mμ) in all three vessel shapes when they are at rest. A little light is transmitted when the vessels are shaken, and the amount transmitted is appreciably greater for the B shape than for the A or C, which appear to absorb equally. However, the eye is very sensitive to traces of transmitted light, and comparison of steady rates of photosynthesis in the different vessels led us to the conclusion that with these densities of cell suspension, the fraction of the light absorbed in vessels of all three shapes was so close to 100 % that the differences detected by the eye were negligible.

Although under conditions of near-total absorption the difference between the A and B vessel shapes with respect to light absorption may be negligible, this is not the case with thinner cell suspensions which absorb 50 % or less of the incident light. With thin suspensions, the difference in light absorption during shaking of the A and B shaped vessels may be significant, and as we shall show in a later paper, this difference is probably a contributory source of error in experiments of Warburg (30) with suspensions so thin that the rates of photosynthesis could be computed from steady-state pressure changes in continuous light. These experiments are therefore free from the errors arising from diffusion lag, but again because of the sensitivity of the two-vessel method to small errors, a small inequality in light absorption can lead to a relatively large error in computed rate of photosynthesis.

The light source was a 450 watt mercury-cadmium arc from Phillips Lamp Company. The red cadmium line (644 mμ) was isolated by means of a red-transmitting glass and a heat absorbing glass. Of the energy transmitted by this filter combination, 83 % was found to be in the 644 mμ cadmium line while 17 % was of longer wave lengths, including the mercury line at 691 mμ, with some continuous background radiation from the faint glow of the quartz envelop of the arc, and from the hot electrodes. Most of the 17 % beyond 644 mμ was of wave lengths longer than 700 mμ, chiefly from cadmium and mercury lines between 700 and 1000 mμ. By using a glass which absorbed the 644 mμ line and transmitted the lines of

longer wave length, we were able to show that all the combined energy of wave lengths longer than 644 $m\mu$ produced no detectable photosynthesis. Therefore in calculating efficiencies we considered only the fraction of the energy from the 644 $m\mu$ line (83 %) was available for photosynthesis. All energy measurements were made bolometrically, with a large-surface platinum bolometer, calibrated against a radiation standard from the U. S. Bureau of Standards. For calibration, a fluorite window was used. This window was tested by the Bureau of Standards and found to transmit 92 % of the energy emitted by the radiation standard (reflection losses only).

The beams of light incident upon the vessels containing the cell suspensions provided about 1.5 μ einsteins of energy per minute at the 644 $m\mu$ wave length, for each vessel. A thermocouple was used to monitor the lamp output at this wave length during the photosynthesis measurements.

Twin beams were taken from opposite sides of the same lamp, by the optical system shown in Nishimura et al (20, p. 191, fig 4). Duplicate systems of prisms and lenses projected rectangular light spots on the bottoms of the two reaction vessels containing the cell suspensions. The size of the light spots and the length of the shaking path of the vessels were adjusted so that the light spots were always completely intercepted by the suspensions. The illuminated areas were 20×20 mm. Vessels 40 mm long could be shaken over an 18 mm path, and still intercept the entire light beams during shaking.

The light beams were adjusted to give equal absorption of energy for photosynthesis in the pair of vessels to be used for the two-vessel measurements. Hand-formed reaction vessels scatter, reflect, and refract different fractions of the incident beams, so equality of absorbed energy could not be assured simply by making the incident beams equal. It was necessary to adjust the intensities for each vessel pair used. Equality of photosynthesis was the criterion for equality of energy absorption. A suspension of cells was prepared in carbonate-bicarbonate buffer #9, and 7 or 8 ml were pipetted into each vessel. The two vessels were then shaken in a thermostat, and pressure changes were observed. The two light beams thus adjusted to give equal photosynthesis in the two vessels, differed according to bolometric measurements by about 2 %, for some of the pairs of hand-formed vessels.

Since the two equal samples of cells may be assumed to respire at equal rates in darkness, one should expect that in darkness the pressure changes in the two vessels would be inversely proportional to the vessel constants for oxygen (the pressure changes in carbonate buffer experiments being attributed to oxygen exchange alone). Therefore, in order to assure equality of photosynthesis in the two vessels, it should only be necessary to adjust the intensity of the two light beams so that in light also, pairs of pressure increments were inversely proportional to the vessel constants. If we designate the pressure increments in the two vessels by H and h, and use the capital letter for the *larger* of the two pressure changes (which will refer to the vessel with the *smaller* gas space), we may use capitals to designate all quantities for the vessel with smaller gas space, and small letters for the other vessel. The relationships, expressed algebraically, should be as follows: Since in darkness it is assumed that $X_{O_2} = x_{O_2}$, and since $HK_{O_2} = X_{O_2}$ and $hk_{O_2} = x_{O_2}$,

$$\frac{h}{H} = \frac{K_{O_2}}{k_{O_2}}$$

We always observed, however, that for steady-rates of respiration in darkness, h/H proved to be a little smaller than K_{O_2}/k_{O_2}. Whatever the reason for this difference, if the cell samples in the two vessels are equal, and respire at equal rates, then the ratio h/H (which we may call ρ), determined experimentally from steady-rate measurements in darkness, is a better criterion for equality of photosynthesis during illumination, than the calculated ratio K_{O_2}/k_{O_2}. Our procedure, therefore, was to determine ρ experimentally from steady-rate measurements of respiration in darkness, and then to adjust the light beams so that during steady-rate photosynthesis the ratio of the observed increments H and h was also equal to ρ.

We considered several possible reasons why the values of ρ determined experimentally from pressure increments observed in darkness during steady-rate conditions failed to match the calculated value of K_{O_2}/k_{O_2}, and we made experiments to test several hypotheses. The difference is small (of the order of 2 or 3 %) and, if several factors contribute to it, then it is not surprising that no single factor makes a contribution large enough to be clearly identifiable. With the simple constant-volume manometers we never had encountered difficulty in confirming the ratio K_{O_2}/k_{O_2} through measurements of h/H in darkness, but a difference of 2 %, which is easily detectable in cathetometer measurements with differential manometers, might escape notice in the less precise readings of the constant-volume manometers. On the other hand, the calculation of K_{O_2} and k_{O_2} for the differential manometers involves some approximations because of the changing volume due to motion of the manometer fluid. No such approximations enter into the calculation of K_{O_2} and k_{O_2} for the constant volume manometers. But in the case of the differential manometers, when the area of cross-section of the capillaries is small the approximations seem to be too small to account for observed differences between ρ and K_{O_2}/k_{O_2}.

Another possibility is that the carbonate-bicarbonate buffer fails to suppress carbon dioxide exchange as completely as has always been assumed on the basis of the dissociation constants of carbonic acid, so that the pressure increments H and h represent not only oxygen exchange but some appreciable exchange of carbon dioxide as well. Mass spectrometer measurements made for us through the courtesy of Professor Allan Brown at the University of Minnesota, showed that under conditions approximating those in our experiments, quite appreciable changes in carbon dioxide

concentration above carbonate buffer mixture #9 could be demonstrated. The buffer appears to suppress only about 90 to 95 % of the carbon dioxide exchange, instead of about 99 %, as has been generally assumed. This indicated fraction of carbon dioxide exchange could account for a portion of the difference between ρ and K_{O_2}/k_{O_2}, but probably not for all of it.

Another factor possibly contributing to the disagreement between ρ and K_{O_2}/k_{O_2} is the hanging of manometer fluid on the capillary walls, which makes the downward motion of the meniscus on one side unequal to the upward motion on the opposite side. Any effect due to this factor should vary with the rate of travel of the meniscus, but the difference between ρ and K_{O_2}/k_{O_2} seemed to be independent of rate of travel.

Regardless of what may be the source of the discrepancy, there can be no doubt that ρ_{dark}, measured experimentally, is a better basis for adjusting the light beams for equality of photosynthesis in the two vessels, than the calculated value of K_{O_2}/k_{O_2}. The criterion for equality of photosynthesis is:

$$\rho_{dark} = \rho_{light}$$

Except for the measurements shown in figure 5, all experiments were made at 20° C. Warburg has reported that this temperature was favorable for demonstrating maximal efficiency (Warburg and Burk, 31, p. 429).

The basic equations for calculating gas exchange from two-vessel measurements are the same whether the pressure measurements are made with differential or constant volume manometers. When large numbers of calculations of rates are to be made from pressure measurements, the function ρ serves a useful purpose. In two-vessel experiments, where the pressure increments represent both oxygen and carbon dioxide, the conditions: $X_{O_2} = x_{O_2}$ and $X_{CO_2} = x_{CO_2}$ must both be fulfilled for each pair of values of H and h. For each pair meeting this requirement there is a pair of factors, F_{O_2} and F_{CO_2}, by which H can be multiplied to give X_{O_2} and X_{CO_2}:

$$X_{O_2} = F_{O_2}H$$
$$X_{CO_2} = F_{CO_2}H$$

F_{O_2} and F_{CO_2} are linear functions of the ratio ρ:

$$F_{O_2} = \frac{K_{CO_2}}{\frac{K_{CO_2}}{K_{O_2}} - \frac{k_{CO_2}}{k_{O_2}}} - \rho \frac{k_{CO_2}}{\frac{K_{CO_2}}{K_{O_2}} - \frac{k_{CO_2}}{k_{O_2}}},$$

$$F_{CO_2} = \frac{K_{O_2}}{\frac{K_{O_2}}{K_{CO_2}} - \frac{k_{O_2}}{k_{CO_2}}} - \rho \frac{k_{O_2}}{\frac{K_{O_2}}{K_{CO_2}} - \frac{k_{O_2}}{k_{CO_2}}}$$

The lines can be conveniently plotted from their intercepts.

For F_{O_2} the intercepts are:

$$\text{on } \rho \text{ axis: } \frac{K_{CO_2}}{k_{CO_2}},$$

$$\text{on } F_{O_2} \text{ axis: } \frac{K_{CO_2}}{\frac{K_{CO_2}}{K_{O_2}} - \frac{k_{CO_2}}{k_{O_2}}},$$

For F_{CO_2} the intercepts are:

$$\text{on } \rho \text{ axis: } \frac{K_{O_2}}{k_{O_2}},$$

$$\text{on } F_{CO_2} \text{ axis: } \frac{K_{O_2}}{\frac{K_{O_2}}{K_{CO_2}} - \frac{k_{O_2}}{k_{CO_2}}}$$

Figure 3 shows a plot of these two lines, for a vessel pair found to be equal in diffusion lag for oxygen (the pair tested in fig 8). All the two-vessel experiments reported in this communication were made with this vessel pair. The volumes of the compensating and experimental vessels in ml were as follows:

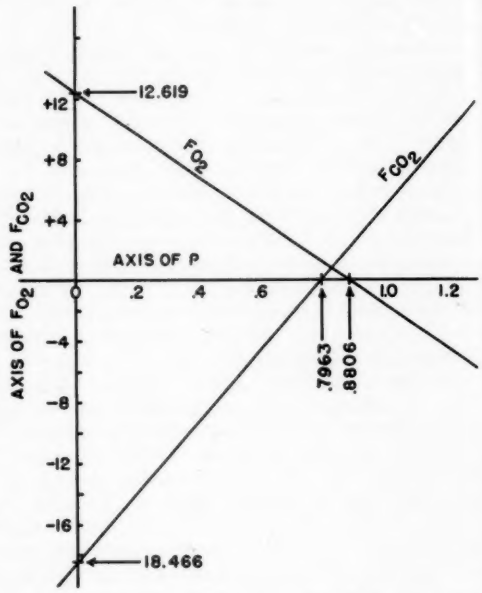

FIG. 3. The lines for F_{O_2} and F_{CO_2} as functions of ρ. These lines represent the equations:

$$F_{O_2} = 14.33\ (0.8806 - \rho)$$
and
$$F_{CO_2} = 23.15\ (\rho - 0.7963).$$

The data from which these equations were derived are:

Manometer for H		Manometer for h	
Experimental vessel	Compensating vessel	Experimental vessel	Compensating vessel
14.94 ml	14.92 ml	18.38 ml	18.46 ml

7 ml fluid in each vessel, 5 % carbon dioxide in air, temperature 20° C, manometer capillary volume 0.00426 ml /cm length.

Shape A vessels	V_F	V_G	Shape C vessels	v_f	v_g
Compensating vessel	7.00	7.92	Compensating vessel	7.00	11.46
Experimental vessel	7.00	7.94	Experimental vessel	7.00	11.38

With a capillary cross-section of 0.426 mm², and with 5 % carbon dioxide in air in both the compensating and experimental vessels, the values of the constants for these vessel combinations are:

$$K_{O_2} = 1.208 \qquad k_{O_2} = 1.517$$
$$K_{CO_2} = 1.955 \qquad k_{CO_2} = 2.220$$

The intercepts plotted in figure 3 were derived from these constants. When the plot is made on a large scale, it is a simple matter to read off for any value of ρ the corresponding values of F_{O_2} and F_{CO_2}.

It is to be noted that the ρ used here is the reciprocal of the one used by Nishimura et al (20). This change was made because their notation leads to equations in which F_{O_2} and F_{CO_2} are not linear functions of ρ, and are less convenient to plot than our linear functions. Our F_{O_2} and F_{CO_2} correspond to their K'_{O_2} and K'_{CO_2}, which we have dropped because they seem too easily confused with K_{O_2} and K_{CO_2}.

Figure 3 illustrates several features of the two-vessel method. It shows that for a certain value of ρ (ca 0.65 for this combination of manometers, vessels, and temperature), F_{O_2} and F_{CO_2} are equal in numerical value and opposite in sign (+3.3 and −3.3). In other words, a value of −1 for γ is characterized by a ρ of 0.65.

The curve for γ as a function of ρ is a rectangular hyperbola (cf. Pirson et al 24, pp. 18–19, figs 6–7). All values of ρ to the left of 0.7963 and to the right of 0.8806 give negative values of γ, and as ρ becomes infinitely negative or infinitely positive, γ approaches an assymptote of about −1.62. At a ρ value of 0.7963, γ is zero. It is positive for ρ values between 0.7963 and 0.8806, becoming infinitely positive as ρ approaches 0.8806, and infinitely negative as ρ passes above 0.8806.

The value of ρ where the line for F_{CO_2} crosses the zero line (0.7963) coincides with a value of $F_{O_2} = 1.208$. This is close to the value of K_{O_2} for carbonate buffer experiments (1.218), and the two would be identical except for the fact that the constants for the differential manometer depend slightly upon the gas mixture. The value of K_{O_2} is figured for air in the case of carbonate buffer experiments, and for 5 % carbon dioxide in air for the two-vessel measurements. Apart from these small differences due to the dependence of constants on the gas mixture, the values of F_{O_2} and F_{CO_2} which coincide with a ρ of 0.7963 (1.208 and zero respectively) are the values which should apply to the measurements in carbonate buffer.

We have explained that when we used carbonate buffer as a medium for experiments to adjust the light beams for equality of photosynthesis in a pair of vessels, we expected to find, for equality of oxygen exchange,

$$\rho = \frac{K_{O_2}}{k_{O_2}}$$

Steady rates of respiration in darkness gave a ρ of about 0.77, while K_{O_2}/k_{O_2} was about 0.80. Among possible explanations for the difference between ρ and K_{O_2}/k_{O_2}, we mentioned that the buffer might permit appreciable carbon dioxide exchange, or, stated in another way, F_{CO_2}, instead of being zero, would have a finite value. Figure 3 shows the finite value that would have to be attributed to F_{CO_2} to account for a ρ of 0.77 instead of 0.80. It indicates that F_{CO_2} would have to be about 1/5 the value of F_{O_2}. It seems unlikely that the buffer should permit such large changes in carbon dioxide pressure, so we concluded that this factor alone could not explain the difference between ρ and K_{O_2}/k_{O_2}.

In two-vessel measurements, the values of F_{O_2} and F_{CO_2} depend upon the ratio of the two pressure changes. Small errors in the measurement of either one can therefore lead to disproportionately large errors in F_{O_2} and F_{CO_2}, and to correspondingly large errors in X_{O_2} and X_{CO_2}. The effects of random errors have been discussed by Nishimura et al (20), and more extensively by Pirson et al (24).

Our experiments on diffusion lag with single vessels (fig 5), and our tests of the comparability of diffusion lag in pairs of vessels (figs 7 and 8) were made with cells suspended in carbonate-bicarbonate buffer #9 (15 parts 0.1 M K_2CO_3, 85 parts 0.1 M $NaHCO_3$). Our two-vessel measurements (figs 9–12) were made with cells suspended in acid culture medium (KH_2PO_4) saturated with 5 % carbon dioxide in air (pH about 4.8). Whatever was used as suspending medium for the cells, the same was used in the compensating vessel of each manometer, and the same gas mixtures were used in experimental and compensating vessels.

The cells were prepared for experiments by centrifuging them out of the culture medium, washing them twice in freshly prepared medium, making them up in fresh medium to the desired suspension density, and pipetting 7 or 8 ml into each manometer vessel. For all experiments with acid culture medium, the cell suspensions were saturated with a gas mixture of about 5 % carbon dioxide in air, and the gas spaces and manometer tubes were swept out with the same mixture. Analyses were made of the concentration of carbon dioxide in the mixtures used for the experiments.

Ordinarily the manometers were shaken in darkness for a few minutes, and the rate of pressure change was followed until it was observed to be steady in both vessels, before starting the sequence of light and dark periods. We made experiments with light periods of many different lengths, from one minute up to thirty minutes, usually alternated with dark periods equal to the light periods. For the most part, we used sequences of 10 minutes light–10 minutes dark, repeated several times. One of our objectives was to compare rates of photosynthesis calculated from transient and steady rates of pressure change, and since the transient changes usually lasted five minutes

or longer after each light–dark or dark–light transition, 10-minute periods were about the shortest that could be used to provide a basis for calculation from steady rates. The 10-minute periods make the experiments directly comparable with the tests of the technique shown in figures 5, 7, and 8.

Minor modifications in the culture medium were made from time to time in the course of the work. In general the medium was prepared according to the specifications of Emerson and Lewis (10, p. 818), but in some cases urea was substituted for nitrate as the nitrogen source. We departed from their specifications by omitting $CaCO_3$. Some cultures were grown over incandescent lamps, some over fluorescent lamps. Ofter a reduction in light intensity was made after one or two days' initial growth of the cultures. In general from 10 to 20 μl of cells were inoculated, and cultures were harvested after 5 to 20 fold increase in cell material. Such increases were obtained in from 24 to 72 hours.

Composition of culture medium and conditions during culture growth exert a marked influence upon the transient pressure changes which follow darkening and illumination when the cells are suspended in acid medium saturated with 5 % carbon dioxide (figs 9–12). Emerson and Lewis (11, p. 796) mentioned similar correlations.

In this communication we make no attempt to correlate transient metabolic behavior with culture conditions, because we have been concerned primarily with the demonstration of diffusion lag and its effects in two-vessel measurements. The examples of transient metabolic gas exchange (figs 9 to 12) are chosen to illustrate the range of results obtainable, rather than to demonstrate any special dependence upon culture conditions.

III. The Character of Transitional Pressure Changes, and the Extent to Which Diffusion Lag Is a Contributory Factor

We explained in Part I that time lag due to diffusion may be an important factor in the application of the two-vessel method to measurements of changing metabolic rates. There is no doubt about the presence of a diffusion barrier between the interior of the cells where gas production takes place, and the gas space where the measureable changes in pressure take place. The question is only whether diffusion lag makes a significant contribution to the rates of pressure change during the minutes immediately following changes in illumination.

Disagreement prevails regarding the importance of diffusion lag because under different conditions the transition from one rate of pressure change to another may follow very different courses.

Figure 4 is a diagram showing different courses of transition, which may be observed under different conditions. It shows a steady rate of pressure change of −3 mm per min in darkness. At time t = 0 light is turned on, and photosynthesis, superimposed upon the continuing respiration, brings about a change in the metabolic gas exchange, leading to a new steady rate of pressure change, of +4 mm per min. The solid line shows the kind of transition from one rate to another which we would expect if diffusion lag were a factor of appreciable importance. According to the way the curve is drawn, a period of about 5 minutes elapses from the termination of the steady dark rate at time t = 0 until there is a close approach to the new steady rate in light. Pressure changes observed at intervals of 5 or 10 minutes after t = 0 would show noticeable effects of diffusion lag of this magnitude.

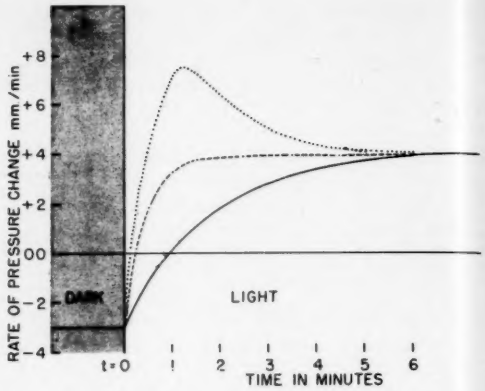

Fig. 4. Diagram showing different courses of transition from a steady dark rate of −3 to a steady light rate of +4 mm per min. The solid line represents the kind of transition observed by Emerson and Lewis for cells suspended in carbonate buffer. The dotted line shows the path of transition they observed for cells in acid phosphate medium. The dashed line is representative of a course of transition corresponding to the behavior described by Warburg and co-workers, for cells suspended in acid culture medium.

The dashed line shows a sharper change in rate. A close approach to the steady rate in light is attained within about one minute from t = 0. If this represented the course of diffusion lag, it would be scarcely noticeable in pressure readings at intervals of 5 or 10 minutes. The effect of such a lag as this would be noticeable if readings were made at intervals of one minute.

The dotted line shows a course of transition which indicates that some process other than diffusion is determining the rate of pressure change. The rate rises to a maximum within the first two minutes after t = 0, and then descends gradually, approaching close to the level of the final steady rate after five minutes. Such a course suggests that besides the steady rate of gas production which leads to the final steady rate of pressure change, there may also be temporary changes in the rate of gas production, to account for the transient maximum. From the dotted curve alone, it would be impossible to judge what might be the contribution of diffusion lag, but it is easy to see that a combination of the diffusion lag represented by the solid line, and some process tending to produce such

a maximum as is shown by the dotted line, would lead to an intermediate course for the rate of pressure change which might approximate the dashed line.

All the three types of transition illustrated in figure 4 have been described in publications reporting measurements of rate of photosynthesis for the purpose of calculating efficiency. The solid curve is representative of the course of transition observable with cells suspended in carbonate buffer. Emerson and Lewis (11, p. 792, fig 5) showed a similar course of transition. Warburg and co-workers have published no data in their recent papers to show the course of transition in carbonate buffer, but there is no doubt that their technique of measurement leads to the appearance of similar behavior. For example, according to Warburg, Burk, and Schade (32, p. 309), "with cells in carbonate . . . transition periods of at least 5 min have to be considered, ..."

Again without producing numerical data, Warburg and co-workers have described transitions similar to the dashed curve in figure 4, for experiments with cells suspended in acid culture medium saturated with 5 % carbon dioxide in air. In describing experiments in which rates of pressure change were observed at intervals of 5 minutes, Burk et al say (6, p. 226) "it was a further improvement owing to this motion that physical transition effects were not observed upon change from dark to light and vice versa, that is, the gas equilibration was virtually perfect for our purposes." A more specific statement, implying transitions which showed even less evidence of diffusion lag than appears in the dashed line of figure 4 reads: ". . . transition effects of equilibration were not ordinarily observed when dark cells were illuminated and vice versa, even when readings were taken every 1 or 2 instead of 5 to 10 min" (Warburg and Burk 31, p. 427).

Rate transitions similar to the dotted line in figure 4 were reported by Emerson and Lewis (10, p. 815; 11, p. 792, fig 2). They attributed the pressure maxima to transient maxima in the metabolic rate of carbon dioxide production ("carbon dioxide burst").

Warburg has referred to the pressure maxima as something peculiar to the experiments of Emerson, but not identifiable as a normal feature of the metabolic activity of the Chlorella cells used in his experiments. He reported (29, p. 202) that he had encountered them at rare intervals, and suggested that they were connected with foaming of the cell suspension (an interpretation which cannot be sustained by either his observations or ours). He has published no discussion of the possibility that the same processes responsible for pressure maxima in our experiments, might have compensated for diffusion lag in his experiments, and made the rate transitions appear to be practically instantaneous. However, after claiming for several years that in his experiments diffusion lag was not appreciable, and that the pressure changes came to steady rates immediately following changes in illumination, he and his co-workers reported sequences of pressure change after changes in illumination which, if they had been plotted as successions of rates (as Emerson and Lewis had plotted their observations), would have looked very similar to the dotted curve in figure 4, and to the pressure maxima which Emerson and Lewis had interpreted as resulting from transient maxima in carbon dioxide production (cf. Warburg, Geleick and Briese 34, pp. 418–419).

Clearly there is a large measure of agreement regarding the transitional rates of pressure change observable under different experimental conditions. In our laboratory, as well as in the laboratories of Warburg and co-workers, it has been observed that when cells are suspended in carbonate buffer a time lag of the order of minutes is observable, while with cells suspended in acid culture medium saturated with about 5 % carbon dioxide in air, instead of a time lag there may be a transient maximum in either positive or negative rate of pressure change.

Disagreement arises over the interpretation of such observations. Warburg and co-workers leave no room for doubt as to their opinion regarding the transient rates. They (Warburg et al, 32, p. 309) write: ". . . it is important to know that in carbonate there is an induction period of minutes until the light action is fully developed manometrically. The cause of this induction is chemical; it cannot be mere physical equilibration, because for the same shaking rates and same conditions there is no corresponding induction if the cells are suspended in acid culture medium."

This statement was published earlier than the papers in which they describe pressure maxima (Warburg, Geleick and Briese, 34; Warburg et al, 35), but there is no evidence that their tardy recognition of the existence of pressure maxima has caused them to modify their belief that diffusion lag is negligible in their experiments, for up to 1954 they continued to treat the sequences of observed pressure changes as if they were quantitatively representative of the rates of gas production in the cells, according to the usual equations of the manometric technique, which presuppose a condition of close approach to equilibrium between gas and liquid phases.

In the publication quoted (32), Warburg refers to the lag in carbonate buffer experiments as "chemical," thus avoiding a direct statement as to whether he regards the lag as physiological. There is no basis for supposing that the establishment of steady states close to equilibria among CO_2, H_2CO_3, and carbonate and bicarbonate ions could lead to time lags of the order of minutes, and other "chemical" causes which would not apply equally to the measurements in acid culture medium seem remote. The only likely alternatives are physiological lag or diffusion lag.

Many years ago Warburg himself observed a time lag in experiments with Chlorella suspended in carbonate buffer (28, p. 192). He investigated this time lag at both high and low intensities. He was able to distinguish between lag due to diffusion, and lag due to physiological factors (so-called "induction"). He concluded that the effects of physiological induction were identifiable at high light intensities, but not at

low intensities. The lag which he observed at both high and low intensities he attributed (correctly, in our opinion) to diffusion. The absence of evidence of physiological induction at low light intensities has been confirmed by many other investigators (recently, for example, by Hill and Whittingham, 14). The increments of light used for measuring efficiency are "low", in the sense that in the dense cell suspensions they bring about average rates of photosynthesis far below the saturation rate, so under the conditions of the efficiency measurements no large effects from physiological induction would be anticipated.

Our new combination of differential manometers and rectangular vessels, and cathetometer for reading pressure changes, makes it possible to follow transitions from one rate to another with a degree of precision which justifies the study of transitional rates under conditions of shaking which approximate those specified by Warburg and co-workers, to see if there is evidence as to the nature of the lag.

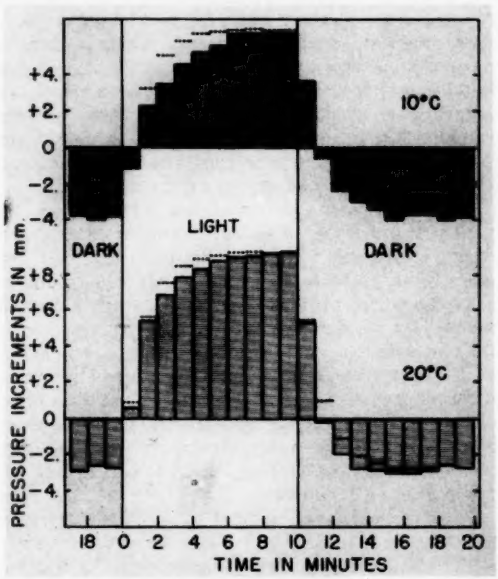

FIG. 5. Pressure increments, observed at one-minute intervals, for dark–light and light–dark transitions, with suspensions of Chlorella in carbonate buffer #9. *Above:* experiment at 10° C, with 260 μl cells in 7 ml of medium, $K_{O_2} = 1.265$. *Below:* experiment at 20° C, with 250 μl cells in 7 ml of medium, $K_{O_2} = 1.208$. Each set of measurements represents the sum of five repeated cycles of 10 min light–10 min dark.

The data for the light-to-dark transition at 10° C were used to derive the value of k for the theoretical curves plotted in figure 6. The fit of the observations for this transition is shown by the open circles plotted in figure 6.

The other three transitions would fit the curves in figure 6 if the succession of increments followed the course indicated by the dotted marks.

Figure 5 shows a record of two experiments, one at 10° C, the other at 20° C. The columns represent increments of pressure change, read at intervals of one minute, for five successive sequences of 10 minutes light–10 minutes dark. The time scale of the figure shows only one sequence of 20 minutes. Each plotted column represents the sum of the five increments observed during the five repetitions of the indicated minute in the sequence. This has the effect of averaging the results of the five cycles. The time scale starts at zero at the beginning of the light period. The increments of the last three dark minutes are re-plotted to the left of time zero, to show the starting point of the transition from dark rate to light rate.

The four sets of transitions appear similar, but they are not identical. If we suppose there is no physiological induction, and that a steady metabolic rate of gas production in either light or darkness is established in cells at the moment of each light–dark or dark–light transition, we may consider what course should be followed by the observable pressure increments if the observed gradual transitions from one rate to another were due to diffusion lag. For this purpose we may treat all rates as positive, measured from a zero line drawn through the average steady rate of respiration, and thus avoid the necessity of formulating our expressions to include both negative and positive rates. If at time $t = 0$ the metabolic rate changes from zero to a steady rate R_∞, and a simple diffusion barrier is all that delays the registration on the manometer of a rate of pressure change commensurate with R_∞, then the rate R_t indicated by the manometer at time t will be related to R_∞ according to the equation

$$R_t = R_\infty(1 - e^{-kt}), \qquad (1)$$

where e is the base of natural logarithms, and k is a constant that includes the various physical characteristics of the system which determine the rate of diffusion of gas from the cells to the gas space. We may call it the apparent diffusion factor of the system. Apart from possible effects of temperature on viscosity of components of the system, we should expect a Q_{10} of some 1.2 for diffusion, so k will be temperature-dependent.

The observations plotted in figure 5 are *increments* from minute to minute, and do not tell us the *rates*, R_t, at the times when the increments were observed, until R_t has become essentially equal to R_∞. To make a direct test of the fit of our data to equation *1* we may let A represent the total accumulated pressure change from time zero to t (time zero representing the start of either a light or a dark period). It can then be shown that

$$R_\infty t - A = (R_\infty/k)(1 - e^{-kt}). \qquad (2)$$

The values of A for successive minutes are derivable from the data plotted in figure 5. R_∞ is the difference between the steady rates of pressure change in darkness and light.

By choosing different values for k, it is possible

to obtain a close fit of equation *2* to each of the four transitions shown in figure 5. The light-to-dark transition at 20° C shows definite evidence of a small maximum in respiration, suggesting that a small accelerating effect of light on respiration might have influenced this sequence of increments. The dark-to-light transition at 10° C is definitely slower than the corresponding one at 20° C, suggesting a small effect of temperature, perhaps no larger than should be anticipated for a diffusion process. However, we recall that although the *average* rate of photosynthesis in the entire dense suspension is well below saturation, the few cells exposed to the full intensity of the incident beam may be exposed to a level of illumination not far from saturation, so that some effect of physiological induction could be anticipated. According to van der Paauw (23, pp. 595–598, fig 16) and also to Hill & Whittingham (14) induction is greater at lower temperatures, and this supports our opinion that induction probably modifies the course of both the dark-to-light transitions. No induction effects are to be anticipated for the light-to-dark transition, and the one at 10° shows no evidence of acceleration of respiration due to previous illumination. Of the four transitions plotted, this one alone shows no clear evidence of complex origin, so we have used it to derive a value of k for the purpose of testing the fit of equation 2 to the observed data.

Figure 6 shows $(R_\infty/k)(1-e^{-kt})$ plotted on a scale of 100, against time in minutes, for a value of 0.78 for k. The solid lines represent the rise and fall of the expression in light and dark, and the open circles represent the values of $R_\infty t - A$ for the light-to-dark transition at 10° C.

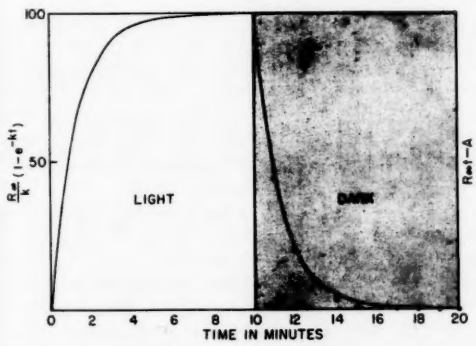

FIG. 6. Comparison of the transitions shown in figure 5 with the expression $(R_\infty/k)(1-e^{-kt})$. The value of k was derived from the observations of the light-to-dark transition at 10° C, shown in figure 5. The open circles show the values of $R_\infty t - A$ derived from these observations. The scale of $(R_\infty/k)(1-e^{-kt})$ is arbitrary. The curves pass the half-way point of upward and downward transitions at about 0.9 min. The transitions shown in figure 5 would fit the course of these curves if the increments followed the sequence of the dotted marks, instead of the observed values.

The amounts by which the other three transitions depart from the course of rise and fall predicted by the curves in figure 6 may be judged from the dotted marks above and below the increments plotted in figure 5. Had the increments come to the levels indicated by the dotted marks, the fit of the experimental values of $R_\infty t - A$ to the curves $(R_\infty/k)(1-e^{-kt})$ would have been exact for all four transitions.

The departures of the increments plotted in figure 5 from the values which would fit the lines plotted in figure 6 for a diffusion process are small, and are easily accounted for in terms of minor effects of light on respiration, a limited effect of physiological induction, and a small effect of temperature on diffusion (for which no allowance was made in calculating the levels of the dotted marks). It could well be that the same diffusion process is the major rate-limiting factor in all four cases.

On the other hand quantitative interpretation of the transitions shown in figure 5 primarily in terms of physiological factors would be inconsistent with certain well established facts regarding photosynthesis. It would require the assumption that cessation of photosynthesis upon darkening follows a slow course nearly symmetrical with the rise after a dark period, and that the induction effects in light were large at low intensities. It would also be necessary to suppose that induction was nearly independent of temperature. If interpretation in terms of physiological induction were rejected, then it would be necessary to assume some rate-limiting chemical process which would have a symmetrical effect for both dark–light and light–dark transitions, and which was furthermore characterized by a temperature coefficient unusually low for chemical processes. Apart from such improbable alternatives, it appears necessary to accept the evidence that under the conditions of these experiments, transitions from one rate of pressure change to another involve a diffusion lag of the order of minutes.

The value of k used for plotting the curves in figure 6 implies a half-time for the change from one rate to another of about 0.9 minute. We have also made direct measurements of diffusion under the same conditions of shaking as were used for the experiment in figure 5, but with water instead of cell suspension in the reaction vessels. Such measurements lead to half-times less than 0.3 min. However in these measurements we have not been able to achieve a degree of precision comparable with that which is attainable in experiments with suspensions of algal cells. The stopping and starting of photosynthesis by repeated alternation of light and dark periods provides a method for repeated reversal of diffusion gradient without interruption of shaking, while direct measurements without cells involve starting each measurement with the manometers and reaction vessels at rest. If there is a concentration gradient between gas and liquid, then when the reaction vessel is at rest the concentration of gas in the liquid phase will not be uniform. There are also other factors which tend to diminish the precision of direct measurements. In

any case, since we are interested in the time lag of the system during the measurements of photosynthesis, cell suspensions are to be preferred, since the presence of the cells increases the viscosity of the liquid. Also, the diffusion barrier between the interior of the cells and the suspending fluid is included, and this may contribute appreciably to the total time lag.

Warburg's argument that there is no diffusion lag in acid culture medium is not conclusive because of the evidence that in acid medium there can be compensatory processes. As we have explained, the transient pressure maxima which are evidence of compensatory processes appear in Warburg's experiments as well as in our own. We find it understandable that the evidence of pressure maxima appears only in the later publications of Warburg and co-workers, because in their earlier experiments they used slower speeds of shaking (for example Burk et al, 6, p. 226, 150/min), while later publications mention the use of higher speeds. (200 per min, Burk and Warburg, 7, p. 14.) Less efficient mixing of the cell suspensions would tend to smooth out the pressure maxima over longer periods and make them less conspicuous. Emerson and Lewis used shaking speeds up to 700 per min in their efforts to make the maxima as clear and sharp as possible.

It is also easy to understand why the maxima appear in acid culture medium saturated with 5 % carbon dioxide, but not in alkaline carbonate buffers. In the case of carbonate buffers, carbon dioxide pressure is maintained at a nearly constant and relatively low level by the buffering action of the carbonate and bicarbonate ions. Changes in pressure are attributable almost entirely to oxygen production. Emerson and Lewis attributed the pressure maxima to transient changes in carbon dioxide production, which would not be observable in carbonate buffer experiments. In the case of acid culture medium, the pressures of both oxygen and carbon dioxide are free to change in response to metabolic production of gases, and the high concentration of carbon dioxide serves to increase the transient maxima. According to the results of Emerson and Lewis, at the low carbon dioxide partial pressures of the carbonate buffers, no prominent maxima in carbon dioxide exchange would be expected, even in acid culture medium.

This review of the evidence shows that there is no difficulty in accounting for the behavior of transitional pressure changes observed in our laboratory and in Warburg's, without recourse to the improbable assumption that diffusion lag was negligible. Among users of the manometric technique, diffusion lag of the order of minutes is so universally recognized and so well supported by experimental evidence (cf Myers and Matsen, 19), that any discussion of it would have been superfluous, were it not for the fact that the repeated statements by Warburg and co-workers that they could find no evidence of diffusion lag led many people to believe that Warburg had devised shaking conditions which practically eliminated diffusion lag.

Having found strong evidence that diffusion lag is a factor of significant magnitude under the conditions of Warburg's measurements of efficiency of photosynthesis, we must next consider what effects it might have upon his application of the two-vessel method, because as we mentioned in Part I, the application of the two-vessel method to periods of transient rates presupposes equality of diffusion lag in the two vessels.

IV. The Testing of Vessel Pairs for Equality of Diffusion Lag

Emerson and Lewis (11), applied the two-vessel method to the study of oxygen and carbon dioxide production during the periods of pressure maxima, and recognized that inequality of diffusion lag in the two vessels would lead to errors. They tried to maintain equality by designing the vessels so that liquid circulation during shaking would be as near alike as possible in the two vessels. But they made no tests of equality of diffusion lag, and emphasized that their results for periods of changing rates should be regarded as approximations, because of the uncertainty about equality.

Warburg and co-workers have recognized the requirement for equality of lag in the two vessels (for example Warburg, Burk and Schade, 32, pp. 307–308), and have stated that in the case of their experiments the requirement was fulfilled, but they have published no evidence from which such a conclusion could be drawn, and it may be doubted whether their method of reading the meniscus levels of manometers with a magnifying glass during shaking at a frequency of 200 per min would permit the comparison of diffusion lag in two different vessels with a degree of precision which would be significant for their application of the two-vessel method.

Nishimura et al (20, p. 200, table 5) tested vessel pairs similar in dimensions to those of Warburg and co-workers, and found clear evidence of inequality of diffusion lag. They also tested pairs of vessels like the A-C combination (fig 2), but came to the conclusion that more precise measurements than naked-eye or hand lens readings of meniscus levels would be required to demonstrate equality of lag.

As far as we are aware, no techniques have been developed for testing the equality of diffusion lag in pairs of manometer vessels destined to be used for two-vessel measurements during periods of transition from one metabolic rate to another. There seems to be no published analysis of the applicability of the method to transitional rates. Pirson's thorough and up-to-date discussion (24) covers steady rates only. There has been no need for a method to test equality of lag, because apart from the work of Warburg and his collaborators, there appear to be no instances where conclusions of wide interest and substantial theoretical importance have been based upon an application of the two-vessel method to the measurement of transitional rates.

Our comparison of diffusion lag presupposes equality of steady-rate respiration and photosynthesis in the two vessels. We have explained (Part II) how we

adjusted the twin light beams to give equal rates of photosynthesis in the two vessels, by first determining the value of h/H, or ρ, during steady rates of respiration in darkness, and then adjusting the intensity of the light beams to give the same value of ρ for steady rates of pressure change in the light. These adjustments were made with dense suspensions of cells, to absorb all the incident light.

These steps to establish equality of steady rates of gas production in the two vessels in both light and darkness depend upon having the cells suspended in carbonate buffer, so that the pressure changes are attributable to oxygen production alone. If the production of two gases contributed to the rates of pressure change, it would be impossible to establish the value of ρ representing equality of gas production in the two vessels, unless γ, the ratio of production of the two gases were known. The need for application of the two-vessel method arises when the value of γ is in doubt.

Similarly, tests of equality of diffusion lag must be made under conditions when the pressure changes represent the production of only one gas, so that there can be no ambiguity as to the significance of the two pressure changes, H and h. If, with cells suspended in carbonate buffer, the light beams have been adjusted to give equality of ρ in both light and darkness, then constancy of ρ as the rate of pressure change changes from one level to another, is a criterion of equality of diffusion lag.

We have made this test by exposing the cells to successive 10-min periods of light and darkness, and observing pressure increments at one-minute intervals. For each pair of increments H and h, we calculated a value of ρ. We show first (fig 7) our application of this test to a vessel pair analogous to the pair described by Warburg and co-workers for their two-vessel measurements (shapes A and B in fig 2).

Pressure changes were read for six successive cycles of 10 min light–10 min dark. The readings of the first cycle are not included in the results. To minimize random fluctuations, the pressure changes for corresponding minutes of the remaining 5 cycles have been summed, as was explained in connection with figure 5.

The top row of columns shows the pressure increments, in mm per min, which represent the values of H. The next row shows the corresponding increments representing h. In each case they are plotted upward and downward from zero.

The last 3 dark minutes are re-plotted at the beginning of the light period, just as was done in figure 5, to show the starting point for the transition to the light rate.

The third row of columns in the figure shows the value of ρ for each minute in the sequence. ρ is defined as h/H, but in the first minutes after light–dark and dark–light changes, it often happens that the increments of h and H are too close to zero to give significant values of ρ. In these cases, instead of calculating from the *increments* of H and h, we have used the *differences* between successive increments.

$$\frac{h_2 - h_1}{H_2 - H_1}$$

When this difference is large compared to the increments themselves, it gives a more significant comparison of diffusion lag than does the pair of increments.

For the vessel pair in figure 7 the steady-rate value of ρ in darkness was 0.53. The light beams had been adjusted so that the same value of ρ was maintained during steady-rate photosynthesis.

Figure 7 shows that after each change in illumination, the values of h approach their new level more promptly than the values of H. This evidence of a difference in diffusion lag is confirmed by the plotted values of ρ, which show systematic departures from the base line of 0.53 during the times of transition from one rate to the other. This indicates that the increments of pressure change in the gas spaces of the two vessels represent unequal amounts of gas during the transition periods. We have explained that for correct application of the two-vessel method, it is essential that the pressure increments always represent equal increments of gas in the two gas spaces. Since this combination of vessel shapes fails to meet the requirement, we must conclude that it is not suitable for two-vessel measurements during transition periods, at least under the conditions of this test.

Whether conditions could be found under which vessel pairs of the A and B shapes would maintain the steady-rate value of ρ during periods of changing rate is a question that should be dealt with by anyone wishing to use such vessel pairs for two-vessel measurements during transition periods. The inequality of diffusion lag shown in figure 7 does not prove that under all conditions such a vessel pair would necessarily show inequality. The evidence shows only that equality should not be assumed, without objective test.

If instead of pairing a vessel of shape B with the A vessel, we use a vessel of shape C for the one with larger gas volume, we shall have more reason to expect equality of diffusion lag, because circulation of the liquid takes place in space of the same dimensions in both vessels. Figure 8 shows a test of an A-C vessel pair, analogous to the test of the A-B pair shown in figure 7. In contrast to figure 7, we see in figure 8 that the values of H and h follow very similar courses of transition from one rate to the other. The values of ρ are plotted upward and downward from a base line of 0.77, the value found for this vessel pair during steady rates of respiration. There is scarcely any evidence of systematic deviations of ρ greater than the random fluctuations. It would hardly be possible to tell from the sequence of ρ values where the light period began and ended.

This test shows that diffusion lag for oxygen may be considered equal in this pair of vessels. Our efforts to devise a correspondingly simple and direct test for

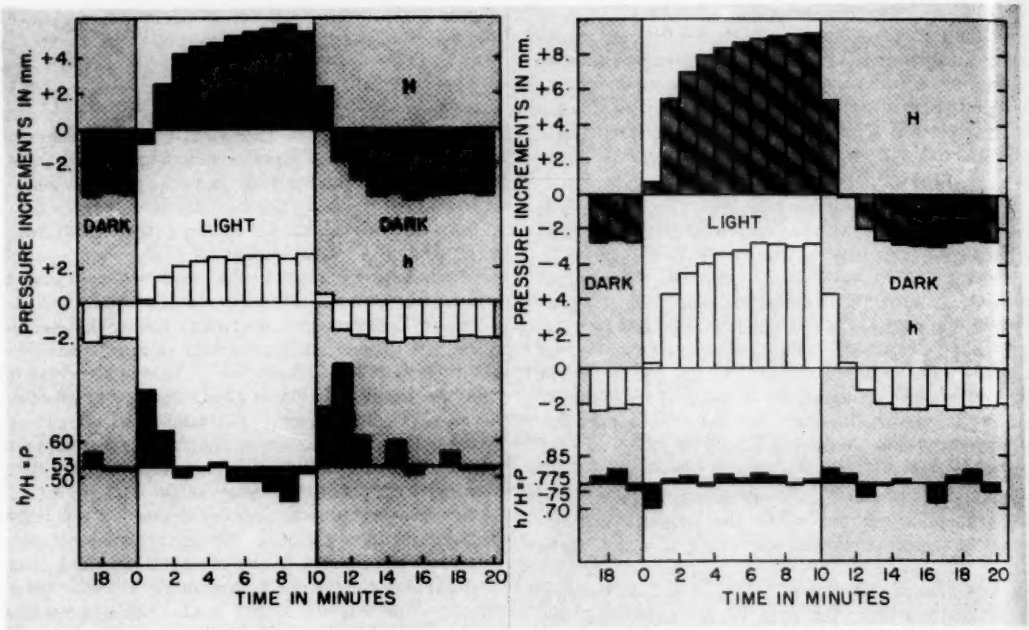

FIG. 7 (*left*). Experiment with carbonate buffer #9, to test equality of diffusion lag for oxygen in a vessel pair similar to the pair described by Warburg and co-workers (shapes A and B in fig 2). The volumes of the vessels containing the cell suspensions were 14.72 and 22.14 ml. Eight ml of suspension and 200 μl of cells were used in each vessel, and K_{O_2} was 1.076, $k_{O_2} = 1.824$. The base line of 0.53 from which the values of ρ are plotted represents the ratio h/H for steady-rate respiration in darkness. The plotted increments of H and h represent the sums of four successive cycles of 10 min light–10 min dark.

FIG. 8 (*right*). An experiment corresponding to the one shown in figure 7, but with a shape C vessel substituted for the shape B vessel, to test equality of diffusion lag for oxygen. Cells were suspended in carbonate mixture #9 again. The volumes of the vessels containing the cell suspensions were 14.94 and 18.38 ml, $K_{O_2} = 1.208$, $k_{O_2} = 1.517$. Seven ml fluid and 250 μl of cells were used in each vessel. The base line of 0.775 from which the values of ρ are plotted represents the value of h/H for steady-rate respiration in darkness. The plotted increments of H and h represent the sums of five successive cycles of 10 min light–10 min dark.

comparison of diffusion lag with respect to carbon dioxide were unsuccessful, and we thought it an open question whether diffusion lag with respect to carbon dioxide would be equal in a vessel pair shown to have equal diffusion lag with respect to oxygen.

After our experimental work was completed, Professor G. E. Briggs showed us that the solubility of the gas enters into the general expression for diffusion lag in such a way that if diffusion lag in a given vessel pair is equal with respect to one gas, it is necessarily unequal with respect to another gas of different solubility, unless the two vessels have equal gas and liquid volumes, a condition which would make the pair useless for two-vessel measurements. The difference in solubility of oxygen and carbon dioxide is so great that the effect of this factor upon diffusion lag cannot be overlooked.

In principle it is therefore impossible to make two vessels equal in diffusion lag with respect to both carbon dioxide and oxygen, and at the same time provide the difference in proportions of gas and liquid volume upon which the application of the two-vessel method depends. This does not diminish the value of the method for the measurement of steady rates, but its application to transient rates is subject to limitation. However, if diffusion lag in the two vessels has been shown to be equal with respect to oxygen, this also establishes the degree of inequality for carbon dioxide. If equality prevails with respect to oxygen, the inequality with respect to carbon dioxide will depend upon the ratio of the constants of the two vessels,

$$\frac{K_{CO_2} k_{O_2}}{k_{CO_2} K_{O_2}}.$$

With the vessel pair listed in figure 8, the predicted difference in diffusion lag for carbon dioxide is only about 10 %. While such a difference may not be negligible, it is nevertheless small enough so that useful information can be obtained from two-vessel measurements with a vessel pair of these characteristics. As we shall show, the method is capable of demonstrating important qualitative differences between the transient rate of gas production in different species

of algae, and in samples of a given species cultured in different ways (figs 9 to 12). It will also be possible to show how rates of photosynthesis leading to very high estimates of efficiency might be derived from two-vessel measurements of transient rates, and to assess the probable significance of the transient rates of gas production and the high estimates of efficiency.

V. Two-vessel Experiments with a Vessel Pair Chosen for Equality of Diffusion Lag for Oxygen

The experiments described in this section were made with the vessel pair used for the test shown in figure 8. It is the same pair for which the values of F_{O_2} and F_{CO_2} are plotted as functions of ρ in figure 3. From time to time in the course of the work, the equality of photosynthesis in the twin light beams was tested according to the method described in Part II. With cells suspended in carbonate buffer, it was verified that the value of ρ for steady rates of pressure change in the light remained equal to 0.77, the value for steady rates of pressure change in darkness. Apart from these tests, the measurements were made with cells suspended in acid culture medium saturated with 5 % carbon dioxide in air. As explained in Part II, under these conditions ρ has a significance different from that for measurements in carbonate buffer. In the case of acid culture medium, changes in ρ no longer imply inequalities in rate of gas production, but instead indicate changes in F_{O_2} and F_{CO_2}, the values by which H is to be multiplied to obtain the indicated rates of oxygen and carbon dioxide production. The base line from which ρ was plotted in the carbonate buffer experiments is of no significance for the experiments in acid culture medium. In the case of the two-vessel experiments it is useful to plot ρ upwards and downwards from the value at which $\gamma = -1$. For the vessel pair used, a ρ of about 0.65 corresponds to a value of -1 for γ, and we have plotted ρ from this base line in the figures 9 to 12.

If no adjustment is made for the great sensitivity of two-vessel measurements to random errors, the fluctuations in increments of calculated gas exchange from minute to minute may sometimes be so great that it is difficult to discern the course of the gas production indicated by the pressure changes. Emerson and Lewis (11) calculated gas exchange from smoothed curves drawn through their observations, instead of from the observations themselves. For the most part we have calculated gas exchange directly from our pairs of observed increments of H and h, but in some cases, especially where H and h are small, there has been an obvious need to smooth out random fluctuations. Where this has been done, dotted marks indicate the adjustments made in the original observations before calculating the values of ρ and the increments of gas exchange. For example in figure 9 it was assumed that in the last five minutes of light the values of both H and h should follow a smooth downward course, instead of the slightly irregular downward course of the observations. Smoothing was done arithmetically, so the total net change was kept identical with the total of the original observations. The dotted lines in the plots of both H and h show the very small extent of the adjustments made. Similar small adjustments may be seen in subsequent figures. No alteration in conclusions nor in calculated rates of photosynthesis results from these adjustments. The totals of the increments of oxygen and carbon dioxide exchange remain the same. The only effect is the smoothing out of random fluctuations in the increments of oxygen and carbon dioxide production, which would be far larger than the apparently trivial fluctuations in H and h.

Figure 9 illustrates the kind of pressure maxima which we have already mentioned as being typical of experiments with acid culture medium. The plotted increments of H and h represent the sums of three successive cycles of 10 minutes light–10 minutes dark. Both sets of increments show a sharp maximum in the first two minutes of light, and a less pronounced maximum in the third, fourth, and fifth minutes of darkness. These maxima are less extreme than those illustrated by Emerson and Lewis, but they are similar in character, and constitute evidence of transient changes in metabolic gas exchange.

Below the increments of H and h are plotted in solid black the values of ρ derived from corresponding pairs of H and h. The last three dark minutes which precede the beginning of the light period show ρ values close to 0.65, indicating approximately equal increments in oxygen and carbon dioxide of opposite sign. In the first three minutes of light ρ rises sharply, then declines gradually toward the end of the light period. In darkness it drops quickly back to 0.65.

The apparent increments in production of the two gases indicated by the pairs of pressure increments are plotted below the values of ρ. After steady rates of gas production have been attained in the cells, and after diffusion between gas and liquid phases has produced a steady state approximating equilibrium between the two phases, then the calculated increments in gas production do actually correspond to the concurrent metabolic increments. During the time of approach to this condition, the calculated increments do not correspond to the metabolic increments. In general, if diffusion lag in the two vessels is not too far from equal with respect to either gas, it may be assumed that the apparent increments will fall short of correctly representing the concurrent changes in metabolic increments. Under special circumstances the apparent increments might for a brief period exceed the actual changes in metabolic increments. But any such errors resulting from inequalities in diffusion lag must be promptly followed by equal errors in the opposite direction, before steady rates of pressure change are re-established. No matter what the errors from minute to minute, as long as the metabolic rates of gas production remain equal in the two vessels, the sum of the calculated apparent increments during the transition period (from the termination of

PRESSURE INCREMENTS IN mm.
H
h
DARK
LIGHT
DARK
h/H=P
INCREMENTS IN GAS EXCHANGE, μ LITERS
=O2
=CO2
TIME IN MINUTES

PRESSURE INCREMENTS IN mm
H
h
DARK
LIGHT
DARK
h/H=P
INCREMENTS IN GAS EXCHANGE, μ LITERS
=O2
=CO2
TIME IN MINUTES

PRESSURE INCREMENTS IN mm.
H
h
DARK
LIGHT
DARK
h/H=P
INCREMENTS IN GAS EXCHANGE, μ LITERS
=O2
=CO2
TIME IN MINUTES

PRESSURE INCREMENTS IN mm.
DARK
LIGHT
DARK
h/H=P
INCREMENTS IN GAS EXCHANGE, μ LITERS
TIME IN MINUTES

one steady rate until close approach to the new steady rate), cannot fail to include, for both oxygen and carbon dioxide, the actual metabolic increments accumulated during the transition period. Also included between the initial and terminal increments of such a period, are the amounts of carbon dioxide and oxygen by which the gas phase fell short of being in equilibrium with the liquid phase at the moment of termination of the preceding steady rate. Missing from the total will be the amounts of the two gases by which gas phase falls short of equilibrium with liquid phase at the time of close approach to establishment of the new steady rate. Allowance can be made for these two effects of diffusion lag, and thus the total increments of oxygen and carbon dioxide during the transition period can be correctly estimated within the limits of accuracy of the method. Departures from a constant ratio of oxygen and carbon dioxide production, or from a steady rate of production of either gas, can therefore be identified with reasonable assurance.

For example in figure 9, we see that the sum total of increments of carbon dioxide between the beginning and end of the light period greatly exceeds the corresponding total of increments for oxygen. The beginning of the light period was preceded by several dark minutes during which the pressure increments in the two vessels were nearly constant, and the corresponding oxygen and carbon dioxide increments were also nearly constant. While a similar constancy has not quite been attained by the end of the light period, the increments are apparently close to attaining steady values. If we assume that the cell samples were equal and maintained equal rates of respiration, and that the twin light beams maintained equal photosynthesis in the two vessels, then there is no escape from the conclusion that at some time during the light period there was a large excess of carbon dioxide production. There remains uncertainty as to the exact course by which this excess was built up, and one can certainly question the significance, for example, of the small maximum in oxygen production shown in figure 9 for the first minute of light, an increment which is clearly out of line with the rest of the sequence.

The same reasoning may be applied to the dark period in figure 9. From the end of the close approach to steady rates which had been attained in the last minutes of the light period, up to the approach to new steady rates at the end of the dark period, there were certainly appreciable maxima in the rates of both carbon dioxide production and oxygen consumption. No doubt their distribution in time was different from that shown by the apparent increments, but in spite of all uncertainties the sums of the apparent increments must match the total metabolic increments, subject only to allowance for diffusion lag at beginning and end of the transition period.

The quantum requirement of photosynthesis that can be calculated from this experiment may be compared with the requirement indicated by the experiments in carbonate buffer, in figure 8, for example. If in both cases we base the calculation upon the more or less steady rates observed in the last few minutes of both light and darkness, we find for the carbonate experiment a requirement of 11.6 quanta per molecule of oxygen, and for the experiment in figure 9 a requirement of 9.6 quanta. In the case of the experi-

FIG. 9 (*upper, left*). Experiment with *Chlorella pyrenoidosa* cells suspended in acid culture medium with 5% carbon dioxide in air, for measurement of transitional changes in oxygen and carbon dioxide exchange. Seven ml of suspension and 295 μl cells were used in each vessel. The plotted increments of pressure and of apparent gas production or consumption represent the sums of three successive cycles of 10 min light–10 min dark. The vessel pair is the one for which the constants are plotted in figure 3.

The random fluctuations in the last five light minutes for H and the last 4 light minutes for h have been smoothed before calculating the plotted values of ρ and the increments of oxygen and carbon dioxide. The smoothing is indicated by the dotted marks. It does not alter the total exchange of either oxygen or carbon dioxide, but leads to a smoother course for the calculated gas exchange during these minutes.

FIG. 10 (*upper, right*). Experiment with *Chlorella pyrenoidosa* cells suspended in acid culture medium with 5% carbon dioxide in air, for measurement of transitional changes in oxygen and carbon dioxide exchange. Seven ml of suspension and 255 μl cells were used in each vessel. The plotted pressure increments represent the sums of 4 successive cycles of 10 min light–10 min dark. The increments of apparent gas production or consumption have been divided by 4, so they represent the average values for the 4 cycles. The constants for this vessel pair are the ones plotted in figure 3.

FIG. 11 (*lower, left*). Experiment with Scenedesmus suspended in acid culture medium with 5% carbon dioxide in air, for comparison of the transitions with those shown for *Chlorella pyrenoidosa* in figures 9 and 10. Some smoothing has been done in the light readings, indicated by the dotted marks. The values of ρ and oxygen and carbon dioxide exchange are based on the smoothed values of H and h. No change is made in the total exchange of oxygen or carbon dioxide.

Seven ml of suspension and 265 μl of cells were used in each vessel. The plotted increments represent the sums of four successive cycles of 10 min light–10 min dark. The constants for this vessel pair are plotted in figure 3.

FIG. 12 (*lower, right*). Experiment with Chlorella ("strain 1") suspended in acid culture medium with 5% carbon dioxide in air, for comparison with *Chlorella pyrenoidosa* and Scenedesmus experiments shown in figures 9, 10, and 11.

Seven ml of suspension and 320 μl cells were used in each vessel. The plotted increments represent the sums of 4 successive cycles of 10 min light–10 min dark. The constants for this vessel pair are plotted in figure 3.

ment in figure 9 we can arrive at a lower quantum requirement by estimating the rate of photosynthesis from the highest positive increment (first minute of light) and he highest negative increment (fourth minute of dark). This leads to a quantum requirement of 6.0. But there is no evidence that the maximum rate of oxygen consumption calculated for the fourth dark minute was prevailing during the minute of light which gave the maximum positive increment, and neither of these increments can be assumed to be correctly representative of the concurrent metabolic rate. The calculated quantum requirement of 6.0 is therefore not identifiable with any metabolic rates that can be shown to have taken place. On the other hand the quantum requirement of 9.6 is derived from nearly steady rates of pressure change, which must therefore be close approximations to the values which would correspond to the steady metabolic rates of gas production.

As for the carbon dioxide "burst," it appears to be much smaller than the bursts described by Emerson and Lewis. The difference is attributable to differences in cultural and experimental conditions. Emerson and Lewis had observed that the carbon dioxide burst was maximal at about 10°, and that it was larger after a long dark period. Our present interest is in the magnitude of the burst under the conditions of Warburg's measurements of efficiency of photosynthesis. The 20° C temperature and the 10-minute dark periods between light exposures would be expected to give a much diminished carbon dioxide burst. The significant conclusion to be drawn from the experiment shown in figure 9 is that the apparent carbon dioxide burst is still identifiable at 20° C, and that it can persist for cycle after cycle of 10 minutes light–10 minutes dark. At a temperature of 10° C, and with long periods of light and darkness, and cells grown according to the specifications of Emerson and Lewis, our present technique gave results in good agreement with theirs.

Without giving supporting figures, Emerson and Lewis mentioned indirect evidence that the magnitude of the carbon dioxide burst depended upon the amount of cell material, while the steady rate of photosynthesis depended on the absorption of light. To test the effect of cell quantity on carbon dioxide burst, we have repeated the experiment shown in figure 9, with 195 μl of cells in each vessel instead of 295. The cells were from the same stock suspension in each case. The smaller quantity of cells still gave practically total absorption of the incident light, and in accordance with expectation we calculated nearly the same efficiency of utilization of light for photosynthesis, on the basis of the last few minutes of light and darkness. However, the extra carbon dioxide produced was nearly proportional to the quantity of cells used. Table I shows a comparison of these figures. The near-proportionality between the amount of extra carbon dioxide and the quantity of cells is in accord with the suggestion put forward by Emerson and Lewis that the extra carbon dioxide comes from

TABLE I

COMPARISON OF EXTRA CARBON DIOXIDE AND QUANTUM REQUIREMENT (ϕ^{-1}, QUANTA ABSORBED PER MOLECULE OF OXYGEN PRODUCED) IN PHOTOSYNTHESIS FOR TWO DIFFERENT QUANTITIES OF CELLS

AMT OF CELLS IN EACH VESSEL	ABSORBED LIGHT ENERGY	PHOTOSYNTHETIC OXYGEN PRODUCTION	ϕ^{-1}	EXTRA CO_2* IN 10 MIN
μl	*micromole-quanta/min*	*micromoles per min*	*quanta/O_2 produced*	μl
195	1.5	0.17	8.8	12.9
295 **	1.5	0.15	10.0	17.5

Each experiment represents the average of 3 successive cycles of 10 min light–10 min dark, and the same stock suspension of cells was used in each case.

* Extra carbon dioxide means the amount in excess of the quantity equal and opposite to the oxygen exchange. The figures represent the average of the three cycles in each experiment.

** Represented in figure 9.

metabolic intermediates. The greater the quantity of cells, the larger the pool of metabolic intermediates available for extra carbon dioxide production. On the other hand since the larger quantity of cells resulted in no appreciable increase in absorbed light energy (the absorption being practically 100 % in each case), the photosynthetic oxygen production should be about the same for both the smaller and the larger concentration of cells. The tabulated figures show that this was the case.

The pressure increments for the light period in figure 9 are in agreement with the results of Emerson and Lewis, to the extent that in both cases the pressure maximum appears to result from a maximum in carbon dioxide exchange. For the dark period, the evidence is in conflict with the results of Emerson and Lewis. Both their results (11, p. 792, fig 2) and ours show a pressure maximum in darkness, but in their case the maximum indicated a deficit of carbon dioxide production (roughly equal to the carbon dioxide burst in the light), while in our figure 9 the dark maximum is associated with maxima in both oxygen consumption and carbon dioxide production.

There may also be apparent maxima in oxygen production during the light period. As we have said, the small maximum indicated for the first minute of light in figure 9 is too small to be significant, but figure 10 illustrates a case in which there is a larger oxygen maximum, lasting for two minutes. This experiment represents four successive cycles of ten minutes light–10 minutes dark, instead of the three cycles represented in figure 9.

Just as in the case of figure 9 we considered the *sum-total* of oxygen and carbon dioxide production between periods of approximately steady rates, so we may look at the corresponding totals in figure 10. In the case of the light period we see that although the excess of oxygen production over carbon dioxide is not as great as was the excess of carbon dioxide in the

light period of figure 9, it is nevertheless large enough to be regarded as evidence that at some time during the light period there was an appreciable excess in oxygen production. Again we must keep in mind that its time course may be different from the time course indicated by the plotted increments.

The apparent carbon dioxide maximum in figure 9 and the apparent oxygen maximum in figure 10 are both the result of roughly similar maxima in the observed pressure increments. But in the case of figure 10 the successive pairs of increments lead to a slower increase in ρ, which does not attain as high a maximum as in figure 9. (Note that the scale for ρ is larger in figure 10 than in figure 9.) This leads to different values for F_{O_2} and F_{CO_2}, and accounts for the difference in increments of gas production calculated from patterns of pressure increments which look roughly similar.

The differences to be seen in figures 9 and 10, with respect to patterns of pressure increments, and with respect to apparent maxima in oxygen and carbon dioxide production, are also evident if the results are calculated from individual cycles of light and darkness, instead of from the summed increments of three or four cycles which are represented in the figures. In each experiment, the individual cycles lead to the same pattern of results as the summed cycles. The differences must therefore be regarded as systematic, and cannot be attributed to the accidental combination of different extremes of random errors. The conditions under which the cells for these two experiments were grown were different. For figure 9, cultures had been grown a short time from a heavy inoculum. About 50 μl cells had been inoculated into each flask, and they had been grown about 18 hours at high intensity, followed by 20 hours at reduced intensity. The increase in cell material had been about five fold. For figure 10, the inoculum had been about 12 μl per flask, the growth for 48 hours at high intensity and 48 hours at reduced intensity, resulting in 24 fold increase. We attribute the differences to be seen in figures 9 and 10 to differences in culture conditions, but we cannot say whether any of these conditions are more important than others in bringing about the difference in results.

The results for the dark period in figure 10 are in good agreement with those for the dark period of figure 9. The value of ρ remains close to 0.65 throughout the dark period, indicating near-equal and opposite increments in oxygen and carbon dioxide. There are apparent maxima for both gases, about four minutes after the beginning of the dark period. As in figure 9, the 10-minute periods of light and darkness appear to be barely long enough to give a close approach to steady rates, in the last two minutes or so of each period.

Because of the greater extremes in oxygen exchange, the experiment shown in figure 10 offers a greater range of possibilities for calculating rates of photosynthetic oxygen production and quantum requirements, than does the experiment in figure 9. First, taking the values of oxygen exchange from the nearest approach to steady rates, during the last two minutes of light and darkness, we find a quantum requirement of 8.3, in reasonable agreement with the value calculated from steady rates in figure 9. The opposite extreme is to calculate from the maximum oxygen production (first light minute) and the maximum consumption (fourth dark minute). From these extremes we can calculate a rate of photosynthetic oxygen production equivalent to one molecule for only 3 absorbed quanta. Warburg and co-workers have reported a number of similar values from calculations based similarly, upon the periods which gave maximum difference in increments of oxygen production between light and darkness. By basing their calculations upon the estimated slopes of rate curves, instead of upon actually observed increments, they arrived at quantum requirements of unity (Warburg et al, 34, pp. 418–419). We remind the reader that during the periods of transition from one rate to another, the calculated increments of gas production cannot be assumed to be equal to the concurrent metabolic rates. It is easily possible that for short periods the latter could attain much higher levels.

Since both figures 9 and 10 illustrate similar pressure bursts in the first minutes of illumination, it might be suggested that these bursts are due to purely physical causes, and have no real counterpart in the metabolic behavior of the cells. The fact that the positive and negative maxima in light and darkness are not symmetrical in pattern is not conclusive evidence against this suggestion, although symmetry of reversible physical effects might be expected on general grounds. Emerson and Lewis (11) mentioned several items of evidence that the pressure maxima were representative of physiological behavior of the cells rather than of physical behavior of the measuring system. In particular they observed that cells grown under different culture conditions showed significant differences in capacity to produce pressure bursts, under identical conditions of light absorption, etc. We show in figures 11 and 12 that cells of different species show even larger and more striking differences. These two figures show experiments identical with the ones represented in figures 9 and 10, except for the substitution of different species of algae. Figures 9 and 10 show experiments with a strain of *Chlorella pyrenoidosa* widely used for efficiency measurements and other experiments on photosynthesis. It is the strain used by Emerson and Lewis, and has been designated as the "Emerson strain" by Whittingham and by Myers. The cells of this strain are small (about 20 to 30×10^6 cells/μl packed volume). Figure 11 shows an experiment with Scenedesmus D^3 (Gaffron's strain), and figure 12 shows a corresponding one with a strain of Chlorella of undetermined species, which we designate simply as "strain 1." Its cells are larger than *C. pyrenoidosa*, and run about 2 to 3×10^6 cells per μl packed volume. According to Dr. Paul Silva, it corresponds closely to the taxonomic description of *Chlorella ellipsoidea*.

The measurements with Scenedesmus (fig 11) show no clear evidence of pressure maxima, in either light or darkness, but it is an open question whether periods longer than 10 minutes would show a decline from the apparently steady light and dark rates indicated for the 10-minute periods. The transitions are definitely sharper than those shown in figure 2 for carbonate buffer experiments, and this constitutes evidence of metabolic maxima, though of much smaller magnitude than the maxima indicated for *C. pyrenoidosa* in figures 9 and 10. Perhaps in Scenedesmus the maxima last longer and do not attain such extremes. The values of ρ show large deviations from the base line of 0.65, but these are limited to periods when the pressure increments are near zero, so that the sensitivity of ρ to random errors is very great, and consequently these fluctuations (which appear to be random rather than systematic) are hardly significant.

The absence of evidence of carbon dioxide maxima in figure 11 is in disagreement with the results of Brown and Whittingham (5), who, presumably using the same strain of Scenedesmus, found appreciable bursts of carbon dioxide in response to illumination. This difference is probably due to differences in culture conditions. We have not attempted to find ways of culturing Scenedesmus which would lead to carbon dioxide bursts.

Figure 12, on the other hand, shows marked pressure maxima and consequent transitional fluctuations in oxygen and carbon dioxide, but they are opposite to the effects observed with *C. pyrenoidosa*. Illumination brings about a maximum in *negative* pressure change, associated apparently with a transient maximum of carbon dioxide consumption, instead of a burst of production. The first minutes of darkness show an increase in positive pressure changes, which indicate a transient peak in carbon dioxide production. There is also evidence of transient changes in oxygen exchange in both light and darkness.

Figures 9, 10, 11, and 12 show such diverse patterns of transitional pressure changes that their physiological origin is hardly to be doubted. Errors due to difference in diffusion lag in the two vessels might obscure the actual course of transient metabolic gas production, but could hardly lead, under one set of physical conditions, to such a range of transient pressure changes as is illustrated in these figures.

Table II shows a comparison of the quantum requirements (ϕ^{-1}, number of quanta required per molecule of photosynthetic oxygen production) that can be calculated from the data of the experiments shown in figures 5, 7, 9, 10, 11, and 12, from different choices of time intervals. The purpose of this table is to compare the values of ϕ^{-1} from apparent maximal and from steady or near-steady rates of gas exchange. For each value of ϕ^{-1}, the table shows the minutes of the light and dark periods chosen as a basis for calculation of photosynthetic oxygen production. In the case of the experiments in carbonate buffer (figs 5 and 7), the *maximal* rates are also the nearest approach to *steady* rates, since the effects of diffusion lag overshadow all other transient effects in this medium. Photosynthetic oxygen production is calculated on a single-vessel basis, pressure changes being multiplied by K_{O_2}. In the case of the experiments in acid culture medium with 5 % carbon dioxide in air (figs 9, 10, 11, 12), photosynthetic oxygen production was calculated on the basis of two-vessel measurements. Reference to the figures will show in each case the character of the pressure increments and apparent gas exchanges during the minutes chosen as a basis for estimation of ϕ^{-1}. The figures show that steady rates of gas exchange were scarcely attained within the ten-minute periods. The nearest

TABLE II

QUANTUM REQUIREMENT (ϕ^{-1}, NUMBER OF QUANTA ABSORBED PER MOLECULE OF OXYGEN PRODUCED) MEASURED AND CALCULATED IN DIFFERENT WAYS

MEDIUM	BASIS OF CALCULATION	FIG NO.	MIN CHOSEN FOR CALCULATION OF RATE		CALCULATED PHOTOSYNTHETIC O_2 PRODUCTION	ABSORBED LIGHT ENERGY	ϕ^{-1}
			LIGHT	DARK			
					micromoles per min	*μ einsteins per min*	*μ einsteins per micromole*
Carbonate buffer #9*	Final or steady rates of pressure change	5, 10°	8, 9, 10	8, 9, 10	0.12	1.45	12.0
		5, 20°	8, 9, 10	4, 5, 6	0.13	1.5	11.5
		7	8, 9, 10	6, 7, 8	0.11	1.45	13.0
Acid culture medium,** 5 % CO_2 in air	Final or steady rates of calculated gas exchange	9	6, 7, 8, 9, 10	6, 7, 8, 9, 10	0.15	1.5	10.0
		10	9, 10	9, 10	0.18	1.5	8.3
		11	9, 10	9, 10	0.22	1.3	6.0
		12	6, 7, 8, 9, 10	8, 9, 10	0.11	1.3	11.8
	Maximum calculated increments of gas exchange	9	1	4	0.25	1.5	6.0
		10	1	4	0.50	1.5	3.0
		11	6	6	0.24	1.2	5.0
		12	2, 3	3, 4	0.24	1.3	5.4

* Measurements calculated by single-vessel method.
** Measurements calculated using two-vessel method.

approach to steady rate was used as a basis, usually the final minute or two of the light or dark periods. Maximal rates of photosynthetic oxygen production were calculated from intervals showing maximum difference between oxygen production in light and oxygen consumption in dark. As we have explained, these maxima are only apparent rates, and the true metabolic rates in light and darkness may very likely pass through brief maxima much larger than anything indicated by the measurements. From such rates much smaller values of ϕ^{-1} would be derived. On the other hand in the case of the steady-rate values there is a tendency to overestimate the rates, because the ten-minute periods are not quite long enough for attainment of steady rates, so the values of ϕ^{-1} are more likely to be too low.

For the carbonate buffer experiments, the values of ϕ^{-1} range from about 11 to 13. If allowance were made for some contribution of carbon dioxide to the pressure changes these values might be reduced by as much as 10 percent.

Of the measurements in acid medium, those calculated on a steady-rate basis show values of ϕ^{-1} ranging from about 8 to 12, except for the value of 6 for Scenedesmus. As we have explained periods somewhat longer than 10 min may sometimes be required for approach to steady rates, so perhaps the value of 6 should be regarded as more representative of apparent maximum than of steady rate of oxygen exchange.

VI. Discussion and Conclusions

The results of the experiments described in Parts III and IV hardly call for further comment. In our opinion they leave no doubt that in manometric experiments, even under conditions of rapid shaking, the effects of diffusion lag are easily identifiable. We have shown that for vessel pairs of certain shapes the difference in diffusion lag with respect to oxygen may be large enough so that the use of such pairs for two-vessel measurements involves risk of serious errors in the calculation of rates of gas production from rates of pressure change during periods when the distribution of gases between gas and liquid phases is undergoing change from one steady state to another.

The application of the two vessel method to periods of transient changes in rate can lead to errors of unpredictable magnitude in the rates of both oxygen and carbon dioxide production. Although we have no quantitative information on diffusion lag in the vessel pairs used by Warburg and co-workers, the results of the test shown in figure 7 suggest that with the combination of shapes which they used, differences in lag may have been so great that the calculated rates of oxygen and carbon dioxide production and the derived values of γ could be far from correct. Certainly they do not correspond to the actual rates of metabolic gas production during the time intervals over which the rates of pressure change were measured, although up to the publication of Warburg et al (35), they have been treated as if they did so correspond. Conclusions upon which Warburg and co-workers place especial emphasis (the quantum yield of unity for oxygen production during illumination (7), the capacity of the cell to make up the energy required for photosynthesis by supplementing the energy of the light quantum with energy from oxidative metabolism (34), the equivalence of the oxygen capacity of Chlorella cells and red blood corpuscles (35), etc) are supported primarily by two-vessel measurements which are clearly subject to the limitations we discussed above. These conclusions are, therefore, of doubtful significance.

We have shown that in vessel pairs so shaped that liquid circulation tends to be equal, diffusion lag for oxygen may be equal within the limits of measurement, and we have used such a vessel pair for the two-vessel experiments shown in figures 9 to 12. We explained that after our experimental work was completed, our attention was drawn to the fact that in vessels of unequal volume diffusion lag with respect to one gas implies inequality with respect to a second gas of different solubility. Therefore, no vessel pair can meet the requirements for the two vessel method for oxygen and carbon dioxide during and immediately following changes in metabolic rate of gas production or consumption. However, by establishing equality of diffusion lag with respect to one gas, we have also established the degree of inequality with respect to the other gas, a substantial improvement over inequality of unknown magnitude.

As far as we know, there is no published analysis of the consequences of diffusion lag for two-vessel measurements, such as Roughton (26), for example, has given for single-vessel measurements of the rate of production of a single gas. In collaboration with Professor G. E. Briggs, we plan to prepare such an analysis for publication. Preliminary study shows, however, that calculation of metabolic rates of gas production from changing rates of pressure change (as was done by Roughton for single-vessel experiments) will not be possible in the case of the two-vessel experiments unless the course of transition in rate of production of one of the two gases can be mathematically specified.

Present evidence indicates that there may be transient fluctuations in the metabolic rate of production of both oxygen and carbon dioxide, so there is little prospect that we shall soon be able to specify the transient course for one gas with sufficient accuracy to calculate the course for the other.

The application of the two-vessel method to the study of transient metabolic rates is therefore an approximation. All experimental methods are to some extent approximations, and although this one may be fairly rough we should not dismiss it on that account without first considering whether alternative methods promise to be closer approximations. The transient maxima in rates of pressure change are observable beyond all doubt, and we think there is also no room for doubt that they are of physiological origin. If better and more direct methods can tell us more about these maxima than we can learn through the

application of two-vessel manometry, then there may be no need to make further application of the manometry, except, perhaps, for the measurement of rates under steady-state conditions.

The trouble is that for the most part, the direct methods which are applicable to the special conditions under which the pressure maxima appear, give little or no evidence of transient changes which could account for the pressure maxima.

We recall that considerable evidence of transient maxima in gas exchange has been produced by certain direct methods (for example, in the work of Blinks and Skow, 2; Aufdemgarten, 1; Gaffron, 13 and McAlister, 16). We attempt no complete listing of such observations nor comparison of the methods because for the most part the organisms used have been so different from the unicellular alga Chlorella (higher plants or multicellular algae in many cases), and the conditions of the experiments have been so different from those which we recognize as necessary to produce pressure maxima, that we could hardly claim the effects have a common origin. On the other hand direct methods applied to *Chlorella pyrenoidosa*, under conditions more or less approximating those during which pressure maxima have been observed, have resulted in surprisingly little evidence of transient maxima. Transient changes in the carbon dioxide production of Chlorella were observed by McAlister and Myers (17) and also by van der Veen (27). In the work of McAlister and Myers, the light intensity and carbon dioxide concentration were rather different from the range of these factors in our experiments, but there is a possibility that the effects have something in common. In the case of van der Veen's experiments the conditions were quite similar to ours, and one might regard the results as confirmatory. But experiments of Manning, Daniels, Moore, and others, with the dropping mercury electrode (cf. references listed by Moore and Duggar, 18) showed no indication of maxima in oxygen exchange. Yuan et al (38) used a specially constructed combination of magnetic oxygen analyzer and infrared carbon dioxide analyzer, and searched unsuccessfully for evidence of transient maxima in the exchange of either gas. Brackett et al (3) reported some rather small fluctuations in oxygen exchange, revealed by measurements with a platinum electrode method described by Olson et al (22). Whittingham (37) followed oxygen exchange by a modification of Hill's hemoglobin method, and was unable to confirm the fluctuations reported by Brackett et al (3), or to find any evidence of transient fluctuations in oxygen exchange. Van Norman and Brown (21) used a recording mass spectrometer to follow both oxygen and carbon dioxide exchange during alternations of light and darkness, and reported no evidence of transient maxima. Brown (4) made a more detailed study of oxygen exchange, using isotopes of oxygen to identify the photosynthetic and respiratory exchanges, and reported that respiratory oxygen consumption by *Chlorella pyrenoidosa* followed an essentially constant rate through alternations of light and darkness. The rate of photosynthetic oxygen production during illumination was also steady.

In one case only has a direct method revealed gas exchanges which would clearly lead to pressure maxima such as can be observed manometrically, and this was achieved after several years of unsuccessful effort. Brown and Whittingham (5), after improving the mass spectrometer technique to make it applicable to carbon dioxide assay up to concentrations of 5 %, were able to demonstrate bursts of carbon dioxide production quite analogous to those described by Emerson and Lewis on the basis of their two-vessel manometric measurements. They confirmed the findings of Emerson and Lewis in considerable detail, and also added new and interesting information concerning the carbon dioxide maxima.

There is no corresponding confirmation by direct methods, for such maxima in rate of oxygen production as are indicated in figure 10 for the light period, and in both figures 9 and 10 for the dark period. It is possible that these evidences of transient changes in rates of oxygen production are spurious, and that the pressure maxima are due entirely to carbon dioxide. Emerson and Lewis concluded from their experiments that the rate of oxygen exchange probably followed a steady course, and made smooth transitions between light and dark rates. The equations for effects of diffusion lag in the two-vessel method show that under certain conditions a pressure maximum due to one gas could appear to be representative of the other. In our work we were initially inclined to ascribe the pressure maxima to carbon dioxide, and to regard the evidence of oxygen maxima as doubtful. We cannot specify with certainty the conditions which lead to sequences of pressure increments in the two vessels, from which oxygen maxima can be calculated for the light periods. But although the positive oxygen maximum in the light is elusive, and appears in only a few of our experiments, the negative oxygen maximum in darkness is common to nearly all our experiments, and for the reasons set forth in our discussion of figure 9, we think the manometric evidence is probably significant.

The fact that confirmation by direct methods for the oxygen maxima calculated from manometric measurements has not yet been found, need not be regarded as evidence that these maxima are spurious. In many cases there is doubt as to whether the direct methods could reveal the transient fluctuations in rate indicated by manometry. For example in the method of Yuan et al (38) rapid fluctuations in rate might become lost in the volume of circulatory system required to bring the gas mixture to the analyzing elements of the system. It is perhaps more difficult to understand why Brown, and van Norman and Brown, were unable to find maxima in oxygen with the mass spectrometer, although Brown and Whittingham, using essentially the same method, were able to find full confirmation of the carbon dioxide maxima. We note, however, that the oxygen maxima calculated from our

experiments are considerably smaller than the large carbon dioxide maxima obtainable in light. It was by following the specifications of Emerson and Lewis for producing large carbon dioxide bursts that Brown and Whittingham were just able to demonstrate carbon dioxide bursts with the mass spectrometer. Effects of one tenth this magnitude might well be lost in the random scatter of points in the mass spectrometer readings, whereas they remain large and easily observable in the cathetometer readings of pressure changes for the two-vessel method. Through exchange of information between our laboratory and Brown's, we are led to believe that cells grown in the same manner in both laboratories may show small but easily identifiable pressure maxima although with the mass spectrometer there may be no clear evidence of rate maxima.

The fact that Hill and Whittingham observed no oxygen maxima with the very sensitive and rapid hemoglobin method is also inconclusive, because this technique requires the use of oxygen partial pressures far lower than those prevailing in our manometric experiments, and it might well be that, just as high partial pressures of carbon dioxide are required to demonstrate maxima in rates of carbon dioxide production, oxygen maxima would not appear except at higher partial pressures of oxygen. Also Hill and Whittingham used quantities of cells much smaller than the amounts used in our experiments. We know that carbon dioxide maxima are proportional to quantity of cells, and although we have not demonstrated the same relationship for oxygen maxima we think it likely that this will prove to be the case.

This discussion of the evidence from our own experiments and from those of other investigators in regard to transient maxima makes it clear that as far as carbon dioxide is concerned, there is no doubt that large fluctuations in rate do actually occur. In the case of oxygen the evidence is less convincing, because the manometric results have not yet been confirmed by direct methods. But we think that in all probability the rates of both oxygen and carbon dioxide production undergo large fluctuations immediately following changes in illumination. The evidence suggests that during these changes the rates of gas exchange may not be representative of photosynthesis and respiration in the usual sense. The transient maxima probably represent changes in the size of pools of metabolic intermediates which are maintained at steady levels during steady-rate photosynthesis or respiration, but which must be quickly changed to new levels by any change in the balance between these two processes. This is essentially the interpretation which Franck (12) has proposed for the high efficiencies of photosynthesis reported by Warburg and coworkers. It may be true (as we showed in the discussion of fig 10) that very high yields of oxygen production per quantum of absorbed light may be calculated from the maximum observable difference between transient rates. Reasonable allowance for diffusion lag suggests that rates even higher than those estimated by Warburg could be calculated, leading to yields larger than one oxygen molecule per quantum of light absorbed. But if these high rates represent changes in the size of pools of intermediates, it should be clear that the maximum difference between rates in darkness and light cannot be assumed to represent the rate of oxygen production attributable to the light, because there is no basis for assuming that the processes represented by the negative maximum in darkness were continuing at the same rate during the time of the positive maximum in light. On the contrary, there is every reason for believing that they were not. Apart from this, even if the rate of oxygen production could be shown to attain a temporary maximum indicative of a quantum yield of unity or better, this would not indicate an equivalent efficiency for photosynthesis. Oxygen production from pools of intermediates represents energy changes of quite unknown magnitude, but in all probability far smaller than the energy required for production of carbohydrate and oxygen from carbon dioxide and water, the chemical change which we associate with the term "photosynthesis."

Our experiments offer no clues as to the nature of the intermediates which might constitute the pools from which we suppose the transient maxima are produced. Emerson and Lewis reported (11) that the total extra carbon dioxide production, over and above a carbon dioxide exchange equal and opposite to the oxygen, was sometimes as much as one micromole per 100 μl of cells. Assuming that 100 μl of cells contain about 1 mg of chlorophyll, or roughly one micromole, we see that the extra carbon dioxide may be about the molar equivalent of the chlorophyll. The extra carbon dioxide shown in our table I for the 10-minute light periods of the experiment in figure 9 is only about 6 μl per 100 μl cells in ten minutes, or about 0.25 micromole of carbon dioxide, from a quantity of cells containing roughly one micromole of chlorophyll. Brown and Whittingham (5) reported finding about 0.5 micromoles extra carbon dioxide per μl cells.

These results indicate that the pools of intermediates giving rise to the carbon dioxide maxima attain levels comparable with the concentration of chlorophyll in the cells.

The oxygen maxima generally represent smaller fractions of the volume of cells. In the case of the oxygen maximum in darkness in figure 9, the total excess, over and above the average of the last two minutes, is about 0.04 micromole per 100 μl of cells. The oxygen maximum in darkness in figure 10, calculated on the same basis, represents about 0.2 micromole. The oxygen maximum in the first two minutes of light in figure 10 represents about 0.1 micromole per 100 μl cells. These figures are only approximations, which serve to establish orders of magnitude.

One could consider the possibility that the extra oxygen might represent changes in equilibria between components of the cytochrome system. Davenport and Hill (9, p. 332) have estimated that the amount of cytochrome f present in chloroplasts is about one four-hundredth the amount of chlorophyll. This is so

much smaller than the amounts of extra oxygen estimated from our experiments that the hypothesis is not promising. However, there may be other cytochrome components involved besides cytochrome f, and perhaps oxidation-reduction equilibria outside the chloroplasts contribute something to the maxima.

Finally, this discussion of the interrelations of diffusion lag and transitional rates of gas exchange leads inevitably to the conclusion that measurements of the efficiency of photosynthesis are significant only when they are based upon steady metabolic rates. The results we have reported here support the conclusion reached earlier by a number of other investigators, that a quantum requirement of about 8 per molecule of oxygen produced represents the highest efficiency that can be sustained by the evidence (equivalent to about 30 % in red light). The claims put forward by Warburg and co-workers that from one to four quanta suffice per molecule of oxygen produced, appear to be founded upon experimental methods which cannot be counted upon to give results which are numerically correct, and the results, whether correct or not, cannot be regarded as an appropriate basis for calculating the efficiency of photosynthesis.

Summary

The two-vessel manometric method has sometimes been applied to measurement of photosynthesis during periods of transient rates without proper regard for its limitations. Tests show that diffusion lag is an important factor, and that vessel pairs equal in diffusion lag with respect to oxygen can be selected. Theoretical considerations show, however, that equality with respect to two gases of different solubility is not possible. When the method is applied with vessels matched for oxygen, the results indicate transient maxima in both oxygen and carbon dioxide exchange. If the evidence for oxygen maxima is accepted, then values of ϕ^{-1} (the number of quanta required per molecule of oxygen produced) as low as 3 can be calculated, and actual metabolic rates must represent still lower values. However, evidence is presented which suggests that the transient gas exchanges are not representative of respiration and photosynthesis. Steady-rate measurements, which may be regarded as representative of respiration and photosynthesis, lead to values of ϕ^{-1} from about 8 to 12.

Literature Cited

1. Aufdemgarten, H. Zur Kenntniss der sogenannten Induktionsvorgänge bei der Kohlensäurassimilation. Planta 29: 643–678. 1939.
2. Blinks, L. R. and Skow, R. K. The time course of photosynthesis as shown by the glass electrode, with anomalies in the acidity changes. The time course of photosynthesis as shown by a rapid electrode method for oxygen. Proc. Nat. Acad. Sci., U. S. 24: 413–427. 1938.
3. Brackett, F. S., Olson, R. A., and Crickard, R. G. Respiration and intensity dependence of photosynthesis in Chlorella. Jour. Gen. Physiol. 36: 529–561. 1953.
4. Brown, A. H. The effects of light on respiration using isotopically enriched oxygen. Amer. Jour. Bot. 40: 719–729. 1953.
5. Brown, A. H. and Whittingham, C. P. Identification of the carbon dioxide burst in Chlorella using the recording mass spectrometer. Plant Physiol. 30: 231–237. 1955.
6. Burk, D., Hendricks, S., Korzenovsky, M., Schocken, V., and Warburg, O. The maximum efficiency of photosynthesis: A rediscovery. Science 110: 225–229. 1949.
7. Burk, D. and Warburg, O. Ein Quanten-Reaktion und Kreisprozess der Energie bei der Photosynthese. Zeits. Naturforsch. 6b: 12–22. 1951.
8. Damaschke, K., Tödt, F., Burk, D., and Warburg, O. An electrochemical demonstration of the energy cycle and maximum quantum yield in photosynthesis. Biochim. Biophys. Acta 12: 347–355. 1953.
9. Davenport, H. E. and Hill, R. The preparation and some properties of cytochrome f. Proc. of the Royal Soc. (London) B.139: 327–345. 1952.
10. Emerson, Robert and Lewis, C. M. Factors influencing the efficiency of photosynthesis. Amer. Jour. Bot. 26: 808–822. 1939.
11. Emerson, Robert and Lewis, C. M. Carbon dioxide exchange and the measurement of the quantum yield of photosynthesis. Amer. Jour. Bot. 28: 789–804. 1941.
12. Franck, J. Participation of respiratory intermediates in the process of photosynthesis as an explanation of abnormally high quantum yields. Arch. Biochem. Biophys. 45: 190–229. 1953.
13. Gaffron, H. Studies on the induction period of photosynthesis and light respiration in green algae. Amer. Jour. Bot. 27: 204–216. 1940.
14. Hill, R. and Whittingham, C. P. The induction phase of photosynthesis in Chlorella determined by a spectroscopic method. New Phytologist 52: 133–148. 1953.
15. Manning, W. M., Stauffer, J. F., Dugger, B. M., and Daniels, F. Quantum efficiency of photosynthesis in Chlorella. Jour. Amer. Chem. Soc. 60: 266–274. 1938.
16. McAlister, E. D. Time course of photosynthesis for a higher plant. Smithsonian Misc. Collections 95, No. 24: 1–17. 1937.
17. McAlister, E. D. and Myers, Jack. The time course of photosynthesis and fluorescence observed simultaneously. Smithsonian Misc. Collections 99, No. 6: 1–37. 1940.
18. Moore, W. E. and Dugger, B. M. Quantum efficiency of photosynthesis in Chlorella. In: Photosynthesis in Plants, Franck, J. and Loomis, W. E., ed. Iowa State College Press, Ames, Iowa. 1949.
19. Myers, J. and Matsen, F. A. Kinetic characteristics of Warburg manometry. Arch. Biochem. 55: 373–388. 1955.
20. Nishimura, M. S., Whittingham, C. P., and Emerson, Robert. The maximum efficiency of photosynthesis. Symposia, Soc. Exptl. Biol. 5: 176–210. 1951.
21. van Norman, R. W. and Brown, A. H. The relative rates of photosynthetic assimilation of isotopic forms of carbon dioxide. Plant Physiol. 27: 691–709. 1952.
22. Olson, R. A., Brackett, F. S., and Crickard, R. G. Oxygen tension measurement by a method of time

selection using the static platinum electrode with alternating potential. Jour. Gen. Physiol. 32: 681–703. 1949.
23. VAN DER PAAUW, F. The indirect action of external factors on photosynthesis. Rec. trav. bot. néerl. 29: 497–620. 1932.
24. PIRSON, A., KROLLPFEIFFER, I., and SCHAEFER, G. Leistungsfähigkeit und Fehlerquellen manometrischer Stoffwechselmessungen. Marburger Sitzungsberichte 76: 3–27. 1953.
25. RIEKE, F. F. Quantum efficiencies for photosynthesis and photoreduction in green plants. In: Photosynthesis in Plants, Franck, J. and Loomis, W. E., ed. Iowa State College Press, Ames, Iowa. 1949.
26. ROUGHTON, F. J. W. A method of allowing for the influence of diffusion in manometric measurements of certain rapid biochemical reactions. Jour. Biol. Chem. 141: 129–145. 1941.
27. VAN DER VEEN, R. Induction phenomena in photosynthesis. III. Physiol. Plantarum 3: 247–257. 1950.
28. WARBURG, O. Über die Geschwindigkett der Photochemischen Kohlensäurezersetzung in Lebenden Zellen II. Biochem. Zeits. 103: 188–217. 1920.
29. WARBURG, O. Assimilatory quotient and photochemical yield. Amer. Jour. Bot. 25: 194–204. 1948.
30. WARBURG, O. Messung des Quantenbedarfs der Photosynthese für sehr dünne Zellsuspensionen. Biochim. Biophys. Acta 12: 356–359. 1953.
31. WARBURG, O. and BURK, D. The maximum efficiency of photosynthesis. Arch. Biochem. 25: 410–443. 1950.
32. WARBURG, O., BURK, D., and SCHADE, A. Extensions of photosynthetic experimentation. Symposia, Soc. Exptl. Biol. 5: 306–311. 1951.
33. WARBURG, O., BURK, D., SCHOCKEN, V., and HENDRICKS, S. B. The quantum efficiency of photosynthesis. Biochim. Biophys. Acta 4: 335–346. 1950.
34. WARBURG, O., GELEICK, H., and BRIESE, K. Über die Aufspaltung der Photosynthese in Lichtreaktion und Ruckreaktion. Zeits. Naturforsch. 6b: 417–424. 1951.
35. WARBURG, O., KRIPPAHL, G., SCHRÖDER, W., and BUCHOLZ, W. Sauerstoff Kapazität der Chlorella. Zeits. Naturforsch. 9b: 769–778. 1954.
36. WARBURG, O. and NEGELEIN, E. Ueber den Energiumsatz bei der Kohlensaeurassimilation. Zeits. Phyk. Chem. 52: 235–266. 1922.
37. WHITTINGHAM, C. P. Photosynthesis in Chlorella during intermittent illumination of long periodicity. Plant Physiol. 29: 473–477. 1954.
38. YUAN, E., EVANS, R., and DANIELS, F. Energy efficiency of photosynthesis by Chlorella. Biochim. Biophys. Acta 17: 185–193. 1955.

PASSIVE PERMEATION AND ACTIVE TRANSPORT OF IONS IN PLANT ROOTS[1,2]

EMANUEL EPSTEIN

AGRICULTURAL RESEARCH SERVICE, U. S. DEPARTMENT OF AGRICULTURE, BELTSVILLE, MARYLAND

Active ion transport in plant roots, according to the most widely held view, involves the operation of carriers. The hypothesis is as follows: The ions combine with the carrier molecules and the resulting ion-carrier complexes traverse membranes of limited permeability to the free ions. At the inner surface of the membranes, the ions are released from the carriers. This active process of ion transport depends on metabolism, and is characterized by a high degree of selectivity. Ions taken up by this mechanism are largely nonexchangeable with ambient ions of the same or other species.

The process briefly outlined above is not the only one whereby ions may penetrate plant roots. Cations may be non-metabolically adsorbed on negatively charged surfaces within the root, in stoichiometric exchange for other cations residing on these exchange surfaces. Epstein and Leggett (6) exposed excised barley roots to solutions of radioactive $SrCl_2$ (Sr^*Cl_2). When, after 60 minutes, the roots were briefly rinsed with water and then exposed to a solution of non-radioactive $SrCl_2$ of the same concentration, they lost a large part of their Sr* by exchange. Other cations also displaced exchangeable Sr* from the roots. However, the amount lost to water was only a fraction of the amount lost to salts.

When similar experiments were performed on the absorption of sulfate, from solutions of $K_2S^*O_4$, a large labile fraction was again observed. However, in this instance, the amounts of S^*O_4 lost to water were identical with the amounts lost to non-radioactive sulfate, indicating that no exchanging or displacing ions were needed to effect this removal. It appeared, rather, that a fraction of the S^*O_4 ions in the tissue freely diffused out upon transfer of the tissue to water or salt solutions. Another fraction, however, neither diffused out into water, nor exchanged with ambient non-radioactive sulfate. It will be shown that the former process is a manifestation of an "outer region," "water space," or "apparent free space," to which ions have free and reversible access by diffusion. The second irreversible process is active transport of sulfate which will be discussed in greater detail elsewhere (9). The relation between these two processes will be examined. The findings will be shown to apply also to ions other than sulfate.

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Materials and Methods

Radioactive Solutions: Solutions of potassium sulfate labeled with S^{35} ($K_2S^*O_4$) and of potassium selenate labeled with Se^{75} ($K_2Se^*O_4$) were prepared as described elsewhere (9). Solutions of $KH_2P^*O_4$ (P^{32}) were prepared in the same way as was $K_2S^*O_4$. The procedure for Ca^*Cl_2 (Ca^{45}) followed that described for Sr^*Cl_2 (6). All radioisotopes were obtained from the Oak Ridge National Laboratory.

Radioactive Assay, *Roots:* Roots were assayed for radiosulfate as described elsewhere (9).

Solutions: Aliquots of the solutions were pipetted into circular (3.3 cm in diameter and 8 mm deep), ¼ oz, seamless tin boxes (made by Buckeye Stamping Company, Columbus, Ohio), containing the following: a circle of lens paper, 3.0 cm in diameter; 1 drop of a 5 % solution of "Calgonite" (made by Calgon, Inc., Pittsburgh, Pennsylvania) to act as a spreader; 1 ml of a 0.1 *N* solution of the non-radioactive salt; and 0.2 ml of a 10 % solution of sucrose, which keeps the paper from curling when dry. The samples were dried under infra-red lamps and counted in an internal-sample proportional counter.

Excised Barley Roots: Seeds of barley, var. Atlas 46, were germinated in aerated water for 24 hours, and seedlings grown for 5 days in the dark at 24° C, as described earlier (6), with these two differences: the solution used was Ca $(H_2PO_4)_2$, 2×10^{-4} M, instead of $CaSO_4$, and the plants were left in this solution till the time the roots were rinsed and excised just before the experiment proper, instead of being transferred to water twelve hours earlier.

Standard Experimental Procedure: The roots were prepared and absorption experiments performed essentially as described earlier (6). One gm portions of excised roots were weighed out (after blotting on washed, dry cheesecloth to remove water adhering to the surface) and transferred to 50 ml water in 250 × 22 mm Pyrex test tubes. To initiate the absorption experiment proper, the water was decanted and replaced by 50 ml solution of a salt containing a radioactively labeled ion. The solutions were previously adjusted to pH 4.0 with 0.1 *N* HCl. The shift in pH during the absorption period seldom exceeded 0.1 unit. At the end of the absorption period, the solution containing the radioactively labeled ion was decanted, the roots were removed from their tube by means of a hooked glass rod, blotted on cheesecloth to remove solution adhering to the surface, and transferred to a new tube containing 50.0 ml water or solution of non-radioactive salt. Aeration was continuous, both during the absorption and desorption periods. Following the desorption period, an aliquot of the solution in the desorption tube was taken for assay of the amount of radioactively labeled ion lost by the roots. In some cases, the roots themselves were assayed. All treatments were set up in duplicate, except those of the experiment shown in figure 1, which were not replicated. The following is a summary of the relevant experimental conditions: roots, 1.00 gm fresh weight; volume, 50 ml, temperature, 30° C; pH, 4.0; aeration, continuous.

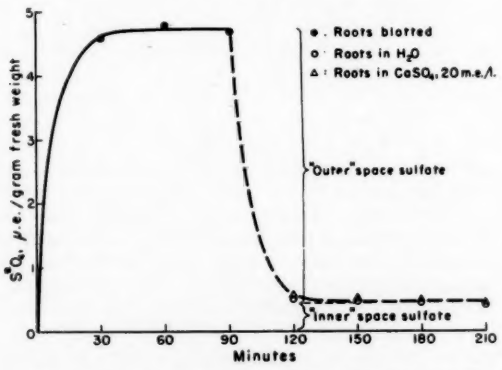


Fig. 1. Uptake and loss of labeled sulfate by excised barley roots. Black circles and solid line: roots in solutions of labeled sulfate ($K_2S^*O_4$, 20 meq/l). Open symbols and broken line: roots in unlabeled media.

Results

Figure 1 presents the results of an experiment on the loss of radiosulfate to water and non-radioactive sulfate. The roots were exposed to a 20 meq/l solution of $K_2S^*O_4$ for 90 minutes, then blotted and transferred to water or a 20 meq/l solution of $CaSO_4$. There occurred an exit of S^*O_4 from the roots which was essentially completed in 60 minutes. A fraction of the S^*O_4 taken up during the initial 90 minutes was retained by the roots, no further loss occurring after the first 60 minutes in water or non-radioactive sulfate. A comparison of the present experiment with that presented in figure 6, Epstein and Leggett (6), shows that the principal difference between the two was that in the latter experiment, only a fraction of the labile Sr* was lost to water, whereas in the present case, the loss of S^*O_4 to water equalled the loss to non-radioactive SO_4. Diffusion, rather than ion exchange seemed to be the factor responsible for this loss. (In preliminary experiments, the roots were briefly rinsed with water, after the absorption period in radiosulfate, in order to remove the solution adhering to the surface of the root. But when it became apparent that diffusion was involved, this procedure was abandoned in favor of blotting.) The findings pointed to the existence, in barley roots, of a region or space to which ions have free and reversible access by diffusion. Terminology and findings for other types of cells and tissues will be considered in the discussion. In this paper, the region accessible by diffusion will be called the "outer" space of the root, and the region to which the ions are transported by the active mechanism, and where they are no longer subject to loss by diffusion or exchange with ambient ions of the same or other ionic species, will be called the "inner" space. On the assumption that at equilibrium, the concentration of the ions in the "outer" space is the same as their concentration in the ambient solution, the "outer" space is defined as

$$\frac{\mu\text{eq diffusible ion/gm fresh wt}}{\text{ambient concentration (in meq/l)}} = \text{"outer" space (in ml/gm fresh wt)}, \quad (1)$$

"ambient concentration" referring to the concentration of the ion during the initial, or absorption, period.

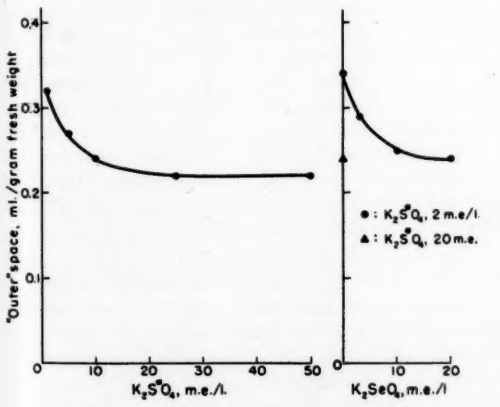
In the experiment shown in figure 1, the amount of S^*O_4 lost to water or non-radioactive SO_4 was 4.45 μeq/gm fresh wt. The concentration of $K_2S^*O_4$ during the initial, or absorption, period, was 20 meq/l, or 20 μeq/ml. Hence the volume of the "outer" space was $4.45/20 = 0.23$ ml/gm fresh wt. The water content of this tissue is 94 %, so that over 24 % of the total tissue water was accessible to the ions by diffusion.

In this experiment, the amount of labile or "outer" space S^*O_4 was determined by the difference in S^*O_4 content between blotted roots and roots which had been held in water or non-radioactive sulfate for 60 minutes or longer. This has the disadvantage that two root samples are required for each determination: a sample of blotted roots for determination of total S^*O_4, and a sample of roots which had been exposed to water or non-radioactive solution for 60 minutes for determination of "inner" space S^*O_4. A different method was therefore used for measuring the "outer" space in the other experiments reported here. The roots were held in the radioactive solution for 60 minutes, blotted, transferred to 50.0 ml water, and after 60 minutes, an aliquot (usually 5 ml) was taken for assay of S^*O_4 lost from the roots. Use of non-radioactive sulfate for the desorbing period was discontinued when the finding shown in figure 1, viz, identical loss to water and non-radioactive sulfate, was verified in many experiments, both for high (20 meq/l) and low (0.5 meq/l) concentrations of S^*O_4 during the absorption period, and of SO_4 during the desorption period.

If ions move into and out of a certain space within the tissue, reaching equality of concentration with the ambient solution, measurement of the volume of this space would not be expected to depend on the particular ambient concentration used (see equation 1). Figure 2 (*left*) shows that this expectation did not seem to be borne out by the experimental findings. While at the high concentrations of $K_2S^*O_4$, the volume of the "outer" space was constant at 0.22 ml/gm fresh wt, it increased at progressively lower concentrations, reaching a value of 0.32 ml at 1 meq/l $K_2S^*O_4$. This finding suggested a labile binding of sulfate which, at low concentrations, would give an apparent or false increase in the measured "space" by contributing to the sulfate released to water. At high concentrations, the amount contributed would be a negligible percentage of the sulfate released, and correct measurements of the "outer" space would result under these conditions.

Experiments were performed at low concentrations of $K_2S^*O_4$, in the presence of high concentrations (20 meq/l) of KCl and KH_2PO_4, in the expectation that excess chloride or phosphate would displace sulfate from the postulated binding sites. It was found, however, that even in the presence of these added anions, the measured "outer" space was still high (of the order of 0.3 ml), when measured by means of $K_2S^*O_4$ at low concentrations (1 or 2 meq/l). However, K_2SeO_4 at increasing concentrations progressively lowered the measured magnitude of the space which eventually reached the same value (0.24 ml in this experiment) obtained when the space was measured with $K_2S^*O_4$ at 20 meq/l, in the absence of K_2SeO_4 (fig 2, *right*). These results are evidence for a labile binding of sulfate and for competition by selenate in this process. So far as measurements of the "outer" space by means of sulfate are concerned, this binding introduces an error by apparently increasing the measured space at low sulfate concentrations.

The above findings suggested a means whereby the "outer" space could be measured without interference by the labile sulfate binding even at low sulfate con-



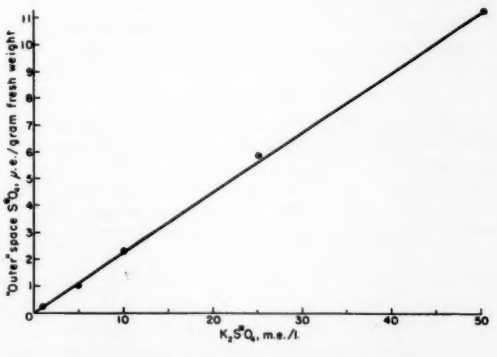


FIG. 2 (*left*). Magnitude of the "outer" space of barley roots as affected by the concentration of $K_2S^*O_4$ used in measuring it (*left*), and by the concentration of K_2SeO_4 at a low concentration of $K_2S^*O_4$ (2 meq/l) (*right*).

FIG. 3 (*right*). Amounts of labeled sulfate in the "outer" space of barley roots as a function of the concentration of $K_2S^*O_4$ in the ambient solution. K_2SeO_4, 20 meq/l throughout.

TABLE I

DETERMINATION OF THE "OUTER" SPACE OF BARLEY ROOTS, USING VARIOUS CONC OF $K_2S^*O_4$

CONC $K_2S^*O_4$	"OUTER" SPACE S^*O_4	"OUTER" SPACE
meq/l	*μeq/gm fresh wt*	*ml/gm fresh wt*
1	0.23	0.23
5	1.02	0.20
10	2.31	0.23
25	5.88	0.24
50	11.33	0.23

20 meq/l K_2SeO_4 was used throughout.

centrations. It was merely necessary to deny the binding sites to sulfate by saturating them with selenate. An experiment was done in which the "outer" space was measured over a wide range of $K_2S^*O_4$ concentrations, in the presence, throughout, of K_2SeO_4 at 20 meq/l. Figure 3 shows that under these conditions, the amount of "outer" space sulfate was strictly proportional to the ambient $K_2S^*O_4$ concentration. In other words, the "outer" space was constant over the entire range of $K_2S^*O_4$ concentrations used (table I).

The "outer" space was found to be independent of the pH of the $K_2S^*O_4$ solution used in measuring it (table II).

It seemed unlikely, on general principles, and in the light of the findings of other workers (1, 8), that diffusion into and out of the "outer" space should be a phenomenon singular to sulfate. Table III presents the results obtained when the space was measured by means of a variety of ions.

The anions were used at a concentration of 20 meq/l. In measuring the space with cations, a complicating factor is the presence, in barley roots, of cation exchange surfaces on which the cations are reversibly adsorbed (6). On transfer of the roots to water, not only will cations diffuse from the "outer" space, but, in addition, a fraction of the cations adsorbed on the exchange surfaces of the root will be lost by hydrolysis (6). This added contribution would tend to give high values for the measured space. For this reason, Ca^*Cl_2 was used at a concentration of 1 meq/l, in the presence of $SrCl_2$ at 20 meq/l. The excess Sr would effectively prevent significant quantities of Ca* from occupying the cation exchange surfaces. It was found for all ions that equilibration was complete in 60 minutes, and absorption and desorption periods of 60 minutes were used. Table III shows that the magnitude of the space was essentially independent of the particular ion used in measuring it.

TABLE II

"OUTER" SPACE OF BARLEY ROOTS AS A FUNCTION OF THE PH OF THE SOLUTION (20 MEQ/L $K_2S^*O_4$) MEASURING IT

PH*	"OUTER" SPACE ML/GM FRESH WT.
4.0	0.22
4.5	0.23
5.9	0.20
7.0	0.23
7.7	0.23

* Adjusted as follows: pH 4.0, by means of 0.1 *N* HCl, as in all other experiments. All other samples, 0.05 M NaH_2PO_4–Na_2HPO_4 buffer (final conc). Shift in pH during the experiment was less than 0.1 unit in every case.

DISCUSSION

The findings presented in this paper indicate, for barley roots grown under the conditions of these experiments, that there occurs a movement of ions between the ambient solution and a compartment or space (the "outer" space) within the root occupying approximately 23 % of the total tissue volume. This movement of ions has the following characteristics: 1) After transfer to a new solution, equilibrium is established within 60 minutes; 2) the evidence is consistent with the conclusion that at equilibrium, the concentration of the ions in the "outer" space equals their concentration in the ambient solution; 3) there is no competition among ions for the space; 4) there is no pH effect; 5) the space is accessible to a variety of inorganic ions. It is concluded that ions move into and out of the "outer" space by diffusion. The characteristics of the diffusion of sulfate into and out of the "outer" space serve to distinguish it unequivocally from active transport of the ions into what, for easy contrast, we call the "inner" space. Sulfate taken up by the latter mechanism is non-diffusible and non-exchangeable with ambient sulfate ions (fig 1, and fig 1, Leggett and Epstein (9)). In active transport, no equilibrium is reached in the experiments, sulfate is accumulated against concentration gradients, selenate competes with sulfate, and there is a pH effect (9).

TABLE III

MEASUREMENT OF THE "OUTER" SPACE OF BARLEY ROOTS BY MEANS OF SEVERAL IONS*

ION	CONC OF ION	"OUTER" SPACE
	meq/l	*ml/gm fresh wt*
S^*O_4	20	0.25
Se^*O_4	20	0.22
$H_2P^*O_4$	20	0.24
Ca^*	1	0.24

* The anions were used as the potassium salts, Ca* was present as Ca^*Cl_2. $SrCl_2$ at a conc of 20 meq/l was present in addition to the Ca^*Cl_2. See text for explanation.

Regions to which various solutes have free and reversible access by diffusion have been recognized in a variety of cells and tissues. Conway and Downey (2) have described an "outer region" or "space" of the yeast cell which is fully accessible to a great variety of solutes, including inorganic ions. The space has a water content of approximately one-tenth the whole cell volume. Cowie et al (3), in experiments with *Escherichia coli*, found that K and Na ions rapidly entered the entire "water space" of the cell (approxi-

mately 75 % of the cell volume), reaching equality of concentration with the ambient solution in less than 5 minutes. Entry of the ions into the water space was reversible, and not a function of metabolism. Roberts et al (10), using the same organism, reported similar findings for sulfate, phosphate, manganese, glucose-1-phosphate, fructose-1,6-diphosphate, and amino acids. Whittam and Davies (11) found that a large fraction of the sodium in guinea pig kidney cortex tissue exchanged at very rapid rates and was indistinguishable from the sodium in the spaces outside the cells. On the assumption that the concentration of the ion in the space was the same as in the medium, the "outer space" occupied approximately 50 % of the tissue at 37° C, suggesting that the barrier to the exchange of the rest of the sodium is not the outer cell membrane but may lie within the cell.

Hope and Stevens (8) were the first to recognize this phenomenon in the roots of higher plants (beans) and termed the space accessible by diffusion the "apparent free space" (AFS). Hope (7) examined the relation between the measured space and the concentration of the KCl solution used for measuring it. The "space" progressively increased with increasing concentration of KCl, indicating a Donnan equilibrium between the root cell cytoplasm and the medium. Butler (1), using wheat roots, and chloride, phosphate, and mannitol for measuring the space, found values ranging from 24 to 34 % of the tissue, but there was no trend in the magnitude of the measured space with changes in the concentration of the solute used in measuring it.

It should be recognized that the variety of terminology notwithstanding, the meaning of the terms used is similar, viz., a space accessible to certain solutes by diffusion.[3] When the solute is specified, it is convenient to speak of "sulfate space," "phosphate space," etc. For a general term, we have used "outer" space, following Conway and Downey (2) who apparently were the first to describe such a space[4] in a plant cell (yeast). The term facilitates the distinction between this space and the "inner" space—the region or regions to which the ions are transported by the active carrier mechanism, and where they are no longer subject to loss by diffusion or exchange with ambient ions of the same or other ion species. "Outer" space is defined here strictly in operational terms, no attempt being made to identify it with particular regions within the tissue or cells. The term is meant to convey the conclusion that this space stands in communication with, and correspondence to, the external solution, so far as the test solutes are concerned.

[3] The "water space" in *E. coli* described by Cowie et al (3) has the additional characteristic that its volume equals the total cell water, whereas the other spaces accessible by diffusion discussed here represent only a fraction of the total cell or tissue water.

[4] "Outer" space as viewed here differs from "apparent free space" in that ions restrained by labile binding and exchange effects are separately accounted for, so that the "apparent" contribution to the space is eliminated.

The external solution and the solution in the "outer" space of the tissue form a continuum.

The only alternative to the interpretation of the demonstrated reversible ion movements as due to diffusion would be one based on a labile binding of the ions, which, on transfer of the tissue to water, would dissociate from the binding entities. This possibility deserves careful consideration in view of the fact that with sulfate, such binding was actually demonstrated at low $K_2S^*O_4$ concentrations. The conclusive test that distinguishes between binding and the lack of it is applied by means of experiments on ion competition. Similar ions tend to compete for identical binding entities, but ions do not compete with one another in diffusion through water. The sulfate uptake giving an apparent or false increase in the measured space was diminished by progressively higher concentrations of selenate, and effectively abolished at a selenate concentration of 20 meq/l. The sulfate concentration in the "outer" space, on the other hand, reached equality of concentration with the ambient solution regardless of the selenate concentration. The space was the same when measured with $K_2S^*O_4$ at 20 meq/l, without selenate (fig 2), as when measured at 1 meq/l or 50 meq/l in the presence of selenate at 20 meq/l (fig 3). This test is the justification for considering the increase in the "space" at low sulfate concentrations, in the absence of selenate, as due to a phenomenon different in kind from diffusion into the outer space proper. Before concluding that a certain uptake involves an "outer" or "free" space, the possibility of labile binding should be ruled out.

The labile binding of sulfate giving rise to the spurious high values for the measured space at low concentrations of sulfate should not be confused with active absorption of sulfate. The processes have in common the fact that selenate competes with sulfate, but following active transport into the "inner" space, sulfate is far from labile, neither being lost to water nor subject to exchange with ambient sulfate (fig 1).

We shall finally examine the relation between diffusion of ions in the "outer" space to active transport of the ions (i.e., their transfer into the "inner" space). It is obvious from the results that the effective interface between the ambient solution and the "inner" space cannot be identified with the epidermis. Rather, the conclusion emerges that *the cells of the root, by their active transport mechanism, absorb ions directly from a solution already within the tissue, viz, the solution in the "outer" space, which, in turn, is in equilibrium with the ambient solution.* Evidence for this conclusion is presented in figure 4.

Following a 60-minute absorption period in $K_2S^*O_4$ at 20 meq/l, the roots were blotted and transferred to tubes containing 50.0 ml water. *They were not, however, placed in the water, but in the space above the water, whereupon the tubes were stoppered.* The roots were thus confined in a small volume (50 ml) of humid air, and could neither gain nor lose sulfate. Immediately after the transfer, and at hourly intervals thereafter, samples were transferred into the

water, and after 60 minutes, aliquots were taken for determination of "outer" space sulfate, and the roots themselves were assayed for "inner" space sulfate. The roots steadily gained "inner" space, or "absorbed" sulfate, at the expense of "outer" space sulfate, with no change in total sulfate. The rate of the process corresponds to the rate of active absorption from a solution of $K_2S^*O_4$ at 20 meq/l. By contrast, cations exchangeably adsorbed on the exchange surfaces of the root, in the absence of an external reservoir of the ions in the solution, are absorbed very slowly by the active mechanism, the exchange spots effectively competing with the carriers for the limited supply of ions (Epstein and Leggett (6), fig. 6).

The picture of the "outer" space of plant roots, as it emerges from the studies on diffusion (1, 7, 8, and the present paper), together with the studies on cation exchange (6), is that of a sponge made of

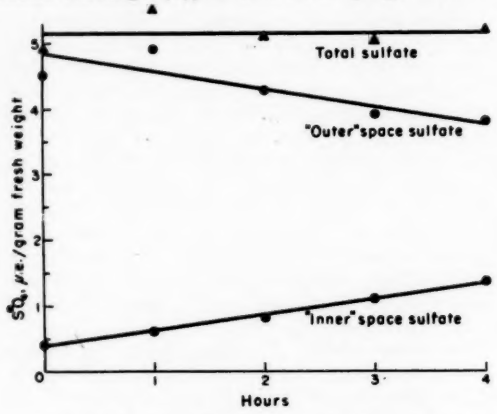


FIG. 4. Transport of labeled sulfate from the "outer" to the "inner" space of barley roots held in humid air. For 60 minutes prior to zero time, the roots were in a solution of $K_2S^*O_4$, 20 meq/l.

cation exchange material. Both anions and cations freely diffuse in the water, and cations exchange on the negatively charged spots of the exchange surfaces. Donnan effects may or may not be pronounced, depending on the concentration of the cation exchange spots, the salt concentration in the ambient solution, and its pH. Within this matrix lie the "inner" spaces, separated from the "outer" space by membranes or barriers across which operate the carriers effecting active ion transport.

The view presented here resolves a paradox apparently inhering in the findings on active ion transport. The evidence and kinetic interpretation presented earlier (4, 5, 6) and in the present paper is that the over-all process of absorption by the active carrier mechanism is essentially irreversible, and that ions so absorbed are nonexchangeable with other ions of the same or other ionic species. How, then, can cells in the interior of the root tissue absorb ions which have previously been absorbed by cells nearer the ambient solution, if this previous absorption was essentially irreversible? Any satisfactory scheme of active ion transport must operate at the cellular and sub-cellular level. Certainly, the process cannot be ascribed to the epidermis cells exclusively—the only cells apparently in direct contact with the ambient solution. The paradox of which we spoke is that of a one-membrane scheme and a multi-membrane tissue.

The evidence for the "outer" space resolves this difficulty by providing for access of the ions to the membranes surrounding the "inner" spaces of the individual cells without requiring them to pass *through* the "inner" spaces of other cells. Rather, the "inner" spaces of the individual cells, through the active carrier mechanism, absorb ions from a common pool—the solution in the "outer" space, which is in equilibrium with the ambient solution.

SUMMARY

Experiments are described demonstrating a passive and reversible permeation of barley root tissue by sulfate and other inorganic ions through diffusion. The volume so accessible (the "outer" space of the tissue), as measured with sulfate, has the following properties. 1) Its magnitude is approximately 23 % of the volume of the tissue. 2) Migration of the ions into this space is freely reversible; upon transfer of the tissue to water, the ions diffuse out of the space again. 3) Equilibration with the ambient medium is reached in 60 minutes. 4) At equilibrium, the concentration of the ions in the "outer" space of the tissue equals their concentration in the ambient medium. 5) There is no competition among different ions for the space. 6) There is no pH effect. The space was freely accessible to selenate, phosphate, and calcium ions, in addition to sulfate.

The relation is examined between diffusion of ions in this "outer" space of the tissue and their absorption by the active ion transport mechanism, i.e., their transfer into the "inner" space. It is shown that the immediate substrate for absorption by the active mechanism is the solution in the "outer" space of the tissue, this solution in turn being in equilibrium with the ambient solution.

LITERATURE CITED

1. BUTLER, G. W. Ion uptake by young wheat plants. II. The "apparent free space" of wheat roots. Physiol. Plantarum 6: 617–635. 1953.
2. CONWAY, E. J. and DOWNEY, M. An outer metabolic region of the yeast cell. Biochem. Jour. 47: 347–355. 1950.
3. COWIE, D. B., ROBERTS, R. B., and ROBERTS, I. Z. Potassium metabolism in *Escherichia coli*. I. Permeability to sodium and potassium ions. Jour. Cellular Comp. Physiol. 34: 243–257. 1949.
4. EPSTEIN, E. Mechanism of ion absorption by roots. Nature 171: 83–84. 1953.
5. EPSTEIN, E. and HAGEN, C. E. A kinetic study of the absorption of alkali cations by barley roots. Plant Physiol. 27: 457–474. 1952.
6. EPSTEIN, E. and LEGGETT, J. E. The absorption of

alkaline earth cations by barley roots: kinetics and mechanism. Amer. Jour. Bot. 41: 785–791. 1954.

7. HOPE, A. B. Salt uptake by root tissue cytoplasm: the relation between uptake and external concentration. Australian Jour. Biol. Sci. 6: 396–409. 1953.

8. HOPE, A. B. and STEVENS, P. G. Electric potential differences in bean roots and their relation to salt uptake. Australian Jour. Sci. Res. B 5: 335–343. 1952.

9. LEGGETT, J. E. and EPSTEIN, E. Kinetics of sulfate absorption by barley roots. Plant Physiol. (In press).

10. ROBERTS, R. B., ABELSON, P. H., COWIE, D. B., BOLTON, E. T., and BRITTEN, R. J. Studies of biosynthesis in *Escherichia coli*. Carnegie Inst. of Wash. Publ. No. 607. Pp. 1–521. 1955.

11. WHITTAM, R. and DAVIES, R. E. Relations between metabolism and the rate of turnover of sodium and potassium in guinea pig kidney-cortex slices. Biochem. Jour. 56: 445–453. 1954.

THE ACTION OF BENZIMIDAZOLE ON LEMNA MINOR[1,2]

WILLIAM S. HILLMAN

DEPARTMENT OF PLANT SCIENCE, YALE UNIVERSITY, NEW HAVEN, CONNECTICUT

Developmental abnormalities induced by chemicals provide a useful approach to the study of plant development if the precise manner in which the formative substance affects metabolism can be established. The experimental object for such a study should preferably be capable of growth under aseptic conditions, morphologically simple so that formative effects may be easily defined, and so constructed that translocation effects of the substance in question are minimized. The common duckweed, *Lemna minor* L., satisfies all these requirements. In addition, its rapid vegetative reproduction insures a large and genetically uniform source of material. A good account of the development of Lemna has been given by Ashby et al (3), and of its growth in aseptic conditions by Gorham (15).

Compounds believed to act as antimetabolites might be expected to exert comparatively easily identifiable biochemical activities. Preliminary tests of a number of such compounds on Lemna showed that benzimidazole (BZ), reported to be an adenine antagonist (32), could induce a profound morphological modification without being toxic (fig 1). This was of particular interest since adenine has been implicated in leaf development and in the action of auxin (4, 28). An investigation of the action of BZ on Lemna was thus undertaken, and developed primarily as a comparison of the physiological characteristics of normal and BZ-modified plants.

MATERIALS AND METHODS

The sterile clone of Lemna used was kindly provided by Dr. Hempstead Castle. Experimental and stock cultures were grown in 125 ml Erlenmeyer flasks containing 50 ml of Gorham's (15) mineral medium. A constant temperature of 20° C was employed, and continuous daylight fluorescent light of 150 to 500 fc intensity, depending on the experiment. BZ was obtained from Krishell Laboratories, Portland, Oregon, and several other sources. It was dissolved by heating with the medium. The pH was adjusted with 0.1 *N* HCl or NaOH before autoclaving, and the change caused by autoclaving, with or without BZ, was 0.1 pH unit or less in the range pH 4.0 to 6.5.

Experimental cultures were started with one "colony" (3 to 4 adhering plants) in each flask and allowed to grow for 10 to 28 days. Multiplication rate, defined by Gorham (15) as the increase in the logarithm (base 10) of plant number per day, of normal plants in the simple mineral medium ranged from 0.060 to 0.150, depending upon light intensity. In order to avoid arbitrary judgments all visible outgrowths from older plants, no matter how small, were counted when plant numbers were determined. Several replicate flasks, usually five, were used for each experimental treatment.

Respiration experiments and enzyme assays were carried out using standard manometric techniques (31) at a water-bath temperature of 27° C. For the respiration experiments, healthy plants were selected, randomized, and transferred with care to Warburg vessels, 50 or 100 being placed in the main chamber (volume about 15 ml) of each vessel with 2 or 3 ml of Gorham's medium. Two-tenths ml of 20 % KOH or of 60 % diethanolamine with folded filter paper in the center-well was used to absorb CO_2. The sensitivities of normal and BZ-modified plants to the respiratory inhibitors cyanide, azide, carbon monoxide, phenylthiourea (PTU) (10, 14) 2,4-dinitrophenol and iodoacetate were compared; comparisons were based on the relative change of the oxygen uptake of each lot of plants from its rate before treatment. Cyanide was used by removing the vessels from the manometers and adding the solution after the control period. This was done to prevent volatile HCN from acting prematurely. It was found that when 60 % diethanolamine (DEA) was used in the center-well instead of KOH the absorption of HCN, and thus the diminution of the cyanide inhibition with time, could be almost abol-

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[2] Taken in part from a dissertation presented to the Faculty of the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ished. All other inhibitors were tipped from the sidearm. PTU was used in saturated solution by tipping a few crystals from the sidearm.

For enzyme assays, plants were thoroughly washed with distilled water, blotted and weighed, then homogenized with a Potter-Elvehjem glass homogenizer or mortar and pestle in an icebath. The crude homogenate was strained through several thicknesses of cheesecloth and stored in ice until use, which was usually within the hour. Enzyme activities were compared on a fresh weight basis. Most of the assays were conducted with 10 to 20 % (fresh weight) homogenates of normal or BZ-modified plants prepared in 0.1 M phosphate buffers or in phosphate buffers with 0.4 M sucrose. The latter medium has been shown (20) to preserve the activity of cytoplasmic particles, and was thus used in cytochrome oxidase and polyphenol oxidase determinations. Besides these two activities, homogenates were tested for catalase, glycolic oxidase, and ascorbic acid oxidation. The endogenous oxygen uptake of homogenates was extremely low no matter what medium was used, usually less than 1.0 μl/ml × 20 minutes. All data used are based on the period after final mixing of the reactants during which oxygen uptake plotted against time gave a straight line. The relatively large amount of material required made it impractical to select typical plants as was done in the respiration experiments. Instead, whole cultures were taken, the number of normal or almost-normal plants in a BZ culture several weeks old being negligible.

Ascorbic acid oxidation was tested by tipping solutions of the compound into homogenates, at both pH 7.9 and pH 4.5. Autoxidation was followed on exactly comparable vessels using boiled homogenate (table IV). Polyphenol oxidase activity was determined by the method of Goddard and Holden (14) modified by the addition of 0.4 M sucrose to the buffer. Cytochrome oxidase was assayed by the same method, using cytochrome c (Sigma Chemical Co.) at a final concentration of 1.7 mg/ml in place of catechol. Catalase activity was estimated by the method of Altschul et al (2), and glycolic acid oxidase was assayed according to Clagett et al (8).

Results

Preliminary Observations: When normal Lemna plants were transferred to medium containing 2.54 × 10^{-3} M BZ, the cultures obtained after several weeks' growth were composed largely of plants with abnormally large light green, translucent fronds, and with roots 2 mm or less in length (compared with a normal root-length of 30 to 40 mm). The BZ-modified fronds showed epinastic curvature in such a way as to appear "humpbacked." These characteristics were associated with a multiplication rate 70 to 90 % of that of the controls. Typical normal and modified plants are shown in figure 1.

Varying the light intensity over the range 50 to 500 fc had no effect on the modification. Initial pH of the culture, however, had a pronounced effect.

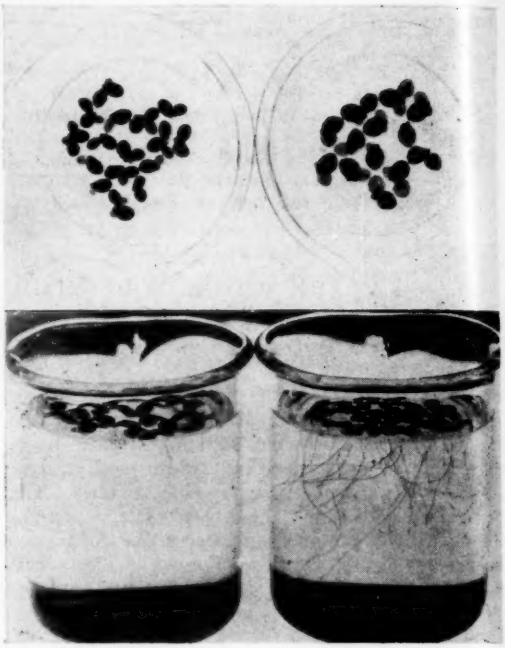

Fig. 1. *Above.* Top view of normal (*left*) and BZ-modified Lemna plants in 100 ml beakers. *Below.* Side view of the same, normal to the right.

With an initial pH of 4.2, a concentration of 6.78 × 10^{-3} M BZ was necessary to obtain the effect given by 8.5 × 10^{-4} M at pH 6.2. Titration curves showed a pK of approximately 5.5 for BZ, indicating that it is most active in the uncharged state, as would be expected for a weakly dissociated compound (27). Since the medium used did not permit prolonged growth with a high initial pH, standard conditions of 3.39 × 10^{-3} M BZ with an initial pH of 5.4 were arbitrarily established for subsequent work, with normal control cultures also started at pH 5.4. At this pH, 4.2 × 10^{-4} M BZ had no visible effect, while 8.5 × 10^{-3} M BZ killed the inoculum plants.

After several weeks' growth, standard BZ cultures such as those described contained not only the typical BZ-modified plants, but also plants in intermediate stages between the normal and modified forms. The earliest plants produced by the normal inoculum were the closest to normal, and so on, 3 to 4 (vegetative) generations in culture being necessary for complete modification. Reports of the occurrence of such intermediate forms are quite common in the literature of formative effects (34) and probably indicate merely that early stages of development are the most sensitive. When completely modified plants were returned to BZ-free medium, a similar but reverse progression of forms to the normal was observed.

The fact that BZ exerted its effect only at concentrations somewhat high for compounds of the growth-factor or hormone type suggested that the modifi-

cation might be due to an impurity in the BZ as supplied, to its partial decomposition upon autoclaving, or to a non-specific buffering effect. However, the activity of BZ was not changed by three recrystallizations, nor by sterilization of the medium by filtration instead of autoclaving. Neither tartrate, phosphate, nor citrate buffer would imitate the action of BZ.

INCREASE IN FROND AREA: Because of the characteristics of culture development described, some form of selection was necessary to obtain typical BZ-modified plants for accurate area estimations. Since only area was under consideration, selection based on other criteria seemed justified. A comparison of the dimensions of normal and modified fronds was made as follows: Standard cultures were grown in 250 fc light for 3 weeks. The criteria for the selection of typical mature plants were, for the normal, a long root and two attached daughter fronds; for the modified, a root 2 mm or less in length and two attached daughter fronds. One hundred plants of each type were removed to beakers. Then from each beaker ten fronds were taken at random and flattened gently. The length from apex to base was measured and also the width, taken as the longest diameter perpendicular to the length. The mean length (± standard error) of the normal fronds was 4.65 ± 0.12 mm, and the width was 3.10 ± .09 mm, giving a length to width ratio of 1.50. The mean length of the modified fronds was 5.90 ± .09 mm, and the width was 4.00 ± .05 mm, giving a length to width ratio of 1.46. Since the ratios of length to width, and the shapes of the fronds, were approximately the same for both types, the products of length and width are related to the true areas by a constant. From calculations of this sort it was possible to show that the increase in area due to BZ was about 65 %.

To determine whether the increase in frond area induced by BZ represents an increase in cell number, the method of Brown and Rickless (6) was used, by which cells of tissues macerated in chromic acid may be counted by standard haemocytometer techniques. Whole fronds were not macerated, since this would introduce extremely variable numbers of cells by the inclusion of young daughter fronds. Instead, distal segments were separated from the apices by cutting through the region of root attachment perpendicular to the longitudinal axis of the fronds. Such segments represent the greater portion of the frond area, and contain only mature tissues. Typical normal and modified plants were obtained as before. Length and width of the segments was determined with an ocular micrometer in a low-power dissecting microscope; since such segments were almost square, the product of these dimensions was close to the true area. For normal fronds, this product was 10.03 mm², for the modified, 17.43 mm² (table I *A*). The maceration data are shown in table I *B*, and indicate no greater cell number in the BZ-modified fronds. One must conclude that the area increase is due to cell expansion.

Water-intake is probably an important factor in the frond area increase. Fresh weight per plant in normal cultures varied from 2.0 to 2.5 mg, while that in BZ cultures was 3.0 mg or higher. In the absence of BZ, frond area and fresh weight was easily affected by variations in the concentration of the medium. If double strength medium was used, frond area was reduced 20 to 40 %. Half- or quarter-strength media caused the production of fronds larger than those in normal strength medium. Corresponding changes in fresh weight were observed, the larger areas being associated with higher fresh weights. When BZ was included in medium already optimally dilute with respect to frond area (¼ to ⅛ strength) it induced no further increase in either fresh weight or area, although the root inhibition and epinasty characteristic of the modified forms were obtained. Fronds grown in dilute medium were already light green and highly translucent; no increase in these characteristics with BZ was noted. The effects of dilute medium cannot be ascribed to mineral deficiencies, since transfer to fresh medium every other day did not moderate the frond expansion observed.

BZ ACTION AND THE EFFECTS OF AUXINS AND ANTIAUXINS: Indoleacetic acid (IAA) or naphthaleneacetic acid (NAA) used in the mineral medium at concentrations of 3 to 6×10^{-6} M induced epinasty and root inhibition resembling those caused by BZ. These levels of the auxins, however, proved toxic; all plants soon died, in contrast with the continued viability of BZ-modified plants. Lower concentrations (ca

TABLE I

CELL COUNTS ON SEGMENTS OF NORMAL AND MODIFIED LEMNA FRONDS: DIMENSIONS OF SEGMENTS AND HAEMOCYTOMETER DATA *

	NORMAL			MODIFIED		
	A	B	C	A	B	C
A. Dimensions of segments in micrometer units (1 unit = 0.135 mm)						
Av length of 10 segments	23.9	23.1	22.0	30.6	30.7	29.6
Av width of 10 segments	24.0	24.2	23.6	31.7	31.9	31.1
Length × width	573.6	559.0	519.0	970.0	979.3	920.0
Av length × width, A, B, C,	550.6 (100 %)			956.4 (174 %)		
B. Haemocytometer counts: Number of cells in standard field						
Counts	36	46	36	27	32	30
	31	33	26	32	30	18
	26	39	31	24	37	28
	35	40	..	28	25	..
	28	28	..	31	40	..
	32	30	..	25	33	..
Av	31.3	36.0	31.0	27.8	32.8	25.3

* Distal segments from plants grown 21 days in Gorham's medium, initial pH 5.4, light 150 fc, temperature 20° C. Modified plants from cultures with 400 mg/l BZ. Each lot of 10 segments macerated in 1.0 ml 5 % chromic acid for 20 hrs at 45° C.

3×10^{-7} M) also caused some increase in frond area, but with slight root inhibition. As with BZ, the area increase occurred only in medium not already optimally dilute. These similarities between BZ and auxin action were suggestive of a common mode of action, but experiments to determine the interactions of BZ with either auxins or antiauxins were complicated not only by pH effects but also by the formative effects of the auxins or antiauxins themselves. 4-Chlorophenoxyisobutyric acid, for example, a reported antiauxin (7), reduced the BZ-induced frond expansion as well as the root inhibition; in the absence of BZ, however, the effective levels (ca 9×10^{-5} M) not only enhanced root growth but induced the production of elongated, occasionally peltate, completely abnormal fronds.

Data indicating a change in auxin relations induced by BZ are shown in figure 2, a comparison of the sensitivities of normal and modified plants to the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) and the closely related antiauxin (19) 2,4,6-trichlorophenoxyacetic acid (2,4,6-T). For the experiment shown, media were prepared containing the indicated concentrations of the compounds; BZ was not present in any of the solutions, so that interference by its buffering effects need not be considered. Two flasks of each medium were then inoculated with two colonies each of normal plants, and two of each medium with modified plants. Results indicate that during recovery from BZ-induced modification the plants showed an increased sensitivity to auxin and a decreased sensitivity to the antiauxin.

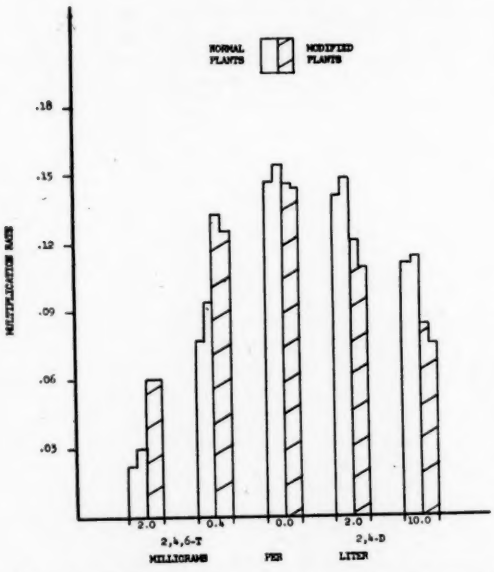

FIG. 2. Multiplication rate, 0 to 6 days, of normal and BZ-modified plants transferred to Gorham's medium containing the indicated concentrations of 2,4-D or 2,4,6-T. Divisions of bars show replicate cultures. Initial pH 5.4, light 450 fc, temperature 20° C.

All experiments with 2,4-D and 2,4,6-T confirmed these results, and both IAA and NAA at suitable concentrations behaved like the 2,4-D. However, neither transcinnamic acid nor triiodobenzoic acid, both often used as antiauxins (5) behaved like 2,4,6-T in exhibiting higher toxicity for normal plants, nor did they discriminate between normal and modified plants.

EFFECTS OF PURINES AND RELATED COMPOUNDS: If the action of BZ on Lemna were due to antipurine activity, one might expect it to be prevented by added purines, or to be imitated by other antipurines. Adenine and guanine were used at concentrations of 3.7×10^{-5}, 3.7 and 7.4×10^{-4}, and 1.48×10^{-3} M in both the presence and absence of BZ. The effects of the purines alone were identical; multiplication rate, increase in frond area, and root elongation were markedly inhibited above 3.7×10^{-4} M, and no effects were observed with 3.7×10^{-5} M. Similar results were obtained in the presence of BZ; no alleviation of the root inhibition caused by BZ was obtained at any concentration. In the three highest concentrations, adenine and BZ together inhibited multiplication rate more than either alone. Comparable results were obtained with adenosine, ribonucleic acid (Schwarz) and desoxyribonucleic acid (Schwarz). Uracil at concentrations from 7×10^{-5} to 9×10^{-3} M failed to reduce the action of BZ.

Four compounds reported to act as purine antagonists: 8-chloroxanthine, 2-aza-adenine, guanazolo, and 2,6-diaminopurine (26) were tested in the mineral medium. All failed to imitate the action of BZ, and all were active at far lower levels. The first inhibited frond expansion above 4.5×10^{-5} M; the next two were toxic above 10^{-6} M, and the last at 10^{-4} M.

RESPIRATION EXPERIMENTS AND ENZYME ASSAYS: Changes in form and physiological activities may be assumed to reflect changes in metabolism. Since oxidative processes are among the most fundamental and frequently studied aspects of metabolism, experiments were undertaken to determine the effects of BZ on respiration. The oxygen consumption of normal plants was found to be unaffected by concentrations of up to 8.47×10^{-3} M BZ tipped from the sidearm. The oxygen consumption of both normal and BZ-modified plants, expressed as μl/100 plants $\times$ hour, ranged from 40 to 60 in various experiments. The rate was linear up to 4 hours in many experiments, but a gradual decline was noted in others. Preliminary results suggesting that the respiratory quotient of modified plants was slightly higher than that of the normal appeared to be of doubtful significance. The inhibitor studies, however, established a clear difference between normal and BZ-modified plants.

The metal-enzyme inhibitors cyanide, azide, carbon monoxide and phenylthiourea (PTU) all discriminated between normal and modified plants, inhibiting the oxygen consumption of the normal but not that of the modified plants. Results of typical experiments with cyanide and with PTU are presented in table II,

TABLE II

THE EFFECTS OF CYANIDE AND PHENYLTHIOUREA (PTU) ON THE OXYGEN CONSUMPTION OF NORMAL AND BZ-MODIFIED LEMNA PLANTS *

50 PLANTS	μL O_2 HR I	INHIBITOR	μL O_2 HR II	% OF HR I	RELATIVE %
Normal	27.6	None	21.4	78	100
Normal	30.7	0.00033 M KCN	15.6	51	65
Normal	26.6	0.003 M KCN	14.4	54	69
Modified	22.1	None	17.0	77	100
Modified	21.9	0.00033 M KCN	20.2	92	119
Modified	21.3	0.003 M KCN	23.0	108	140
Normal	27.0	None	24.8	92	100
Normal	29.2	None	26.4	90	
Normal	24.8	Sat. PTU	18.2	73	79
Normal	27.0	Sat. PTU	19.2	71	
Modified	22.6	None	20.4	90	100
Modified	26.4	None	22.6	86	
Modified	29.2	Sat. PTU	29.7	102	114
Modified	25.8	Sat. PTU	25.3	98	

* Water-bath temperature 27° C. Each vessel contained 2.7 ml Gorham's medium, pH 5.4. KCN solutions (or H_2O) added after hour I. Crystals of PTU tipped from sidearms after hour I. Center well for KCN: 0.2 ml 60 % diethanolamine. Center well for PTU: 0.2 ml 20 % KOH.

and all the inhibitor results are summarized in table III. (Not all the concentrations tried are listed in table III, since lower concentrations had no effect on either type.) While all the metal-enzyme inhibitors discriminated between the normal and modified plants, it is evident that 2,4-dinitrophenol and sodium iodoacetate, which act on other than metal-containing systems (18) did not, but inhibited or increased the oxygen consumption of both types to the same degree. These results indicated a difference between the normal and modified plants with respect to the activity of metal-containing systems, or at least the proportion of oxygen consumption mediated by such systems. Certain concentrations of at least two of the metal enzyme inhibitors, cyanide and azide, actually caused a significant increase in the oxygen consumption of BZ-modified plants.

Since it seemed possible that the difference in sensitivity of normal and modified plants to metal enzyme inhibitors might be due to the lack of roots in the modified plants, experiments on the sensitivity of roots alone were performed. It was found that the sensitivity of normal roots to cyanide and azide was the same as that of the whole normal plants, and that the oxygen consumption of roots constituted about 12 % of the total uptake of a given lot of plants. The difference between the sensitivities of normal and modified plants thus could not be attributed to the lack of roots.

The enzyme assays of homogenates of normal and BZ-modified plants were undertaken to investigate the apparent difference in the activity of metal-containing systems. Typical assays are summarized in table IV, in which the activities of homogenates of normal and modified plants are compared on a fresh weight basis. Polyphenol oxidase activity in homogenates of BZ-modified plants was 4 % or less of the normal activity. Ascorbic acid oxidation was equally low, whether assayed at pH 4.5 or pH 7.9. Cytochrome oxidase activity in homogenates of modified plants was found to be 20, 23, and 33 % of the normal in three assays. Catalase activity in homogenates of BZ-modified plants was 50 to 68 % of normal, and

TABLE III

INHIBITOR SENSITIVITY OF THE OXYGEN CONSUMPTION OF NORMAL AND BZ-MODIFIED PLANTS *

INHIBITOR	CONCENTRATION	APPROXIMATE % INHIBITION (–) OR INCREASE (+) OF O_2 CONSUMPTION	
		NORMAL	MODIFIED
Potassium cyanide	3.3×10^{-4} M	–35	+20
	3.0×10^{-3} M	–35	+40
Sodium azide **	1.0×10^{-3} M	–20	+75
	5.0×10^{-3} M	–50	+10
Carbon monoxide	80 % CO, 20 % air †	–30	+10
1-phenyl-thiourea	Saturated	–20	+15
2,4-Dinitro-phenol	2.0×10^{-5} M	+65	+65
	5.0×10^{-4} M	–20	–25
Sodium iodoacetate **	4.0×10^{-4} M	–10	–25
	2.0×10^{-3} M	–45	–50

* All experiments conducted at 27° C in Gorham's medium, pH 5.4, except as noted. See table II for typical calculations of inhibition or increase.

** pH 4.5 instead of 5.4.

† Control: 80 % nitrogen, 20 % air.

TABLE IV

TYPICAL ENZYME ASSAYS OF NORMAL AND BZ-MODIFIED PLANTS

ASSAY **	ACTIVITIES *		MODIFIED ACTIVITY AS % OF NORMAL
	NORMAL	MODIFIED	
Polyphenol oxidase	45.8	1.7	4
Ascorbic acid oxidation	24.4	1.0	4
Cytochrome oxidase	81.9	19.0	23
Glycolic acid oxidase	55.0	25.0	45
Catalase	64.7	47.2	68

* μl O_2 consumed/gm fresh wt × 20 minutes for all but catalase. All values corrected for autoxidation in boiled homogenates. Catalase as μl O_2 released/20 mg fresh wt × 10 min.

** Systems used: Polyphenolase—increase in O_2 consumption on addition of 0.066 mg/ml catechol to homogenate with 2 mg/ml hydroquinone, 0.4 M sucrose, pH 7.4. Ascorbic acid oxidation—homogenate, 0.25 mg/ml ascorbic acid, pH 7.9. Cytochrome oxidase—like polyphenolase but saturating (1.7 mg/ml) cytochrome c used instead of catechol. Glycolic acid oxidase—like ascorbic acid oxidation, but 1.5 mg/ml glycolic acid instead. Catalase—temperature 0° C, H_2O_2 0.02 %, pH 7.1.

glycolic acid oxidase activity was approximately 50 % of the normal value.

While on the fresh weight basis used all activities were reduced in homogenates of the BZ-modified plants, the virtual absence of polyphenol oxidase and of ascorbic acid oxidation contrasts with the activities of the other enzymes assayed, although the reduction in cytochrome oxidase activity was also marked. Since the first two activities are usually attributable to copper enzymes (18), an effect of BZ on copper metabolism was suggested. This suggestion was strengthened by inhibition studies with PTU, a fairly specific inhibitor for copper enzymes (10). Saturated PTU completely abolished both polyphenol oxidase and ascorbic acid oxidation in homogenates of both normal and BZ-modified plants. In the cytochrome oxidase assays, PTU gave a maximum inhibition of about 10 %; DuBois and Erway (10) had noted none, but inhibitions of as high as 38 % have been reported (14). Neither catalase nor glycolic acid oxidase activities were affected by saturated PTU.

Following this suggestion of a relationship between BZ and copper, it was found that when .05 M solutions of BZ and of copper sulfate were mixed a red precipitate was observed. The precipitate formed only at pH levels higher than 5.5, and redissolved on acidification. These observations led to a thorough search of the chemical literature on BZ, and it was found that a reaction of BZ with cupric salts identical to that described here had been reported in 1919 by Skraup (29), who determined the composition of the precipitate to be $(C_7H_5N_2)_2Cu$. Skraup also found BZ complexes with cadmium, cobalt, nickel, and zinc, but not with iron.

Attempts were then made to cause direct inhibitions of polyphenol oxidase and ascorbic acid oxidation in homogenates of normal plants by tipping in BZ solutions. Up to 8.47×10^{-3} M BZ was ineffective in such experiments but when homogenates were incubated overnight at 0 to 4° C with this concentration of BZ, 20 to 25 % inhibitions were observed on comparison with homogenates incubated without BZ. The inhibitions were observed in experiments conducted at pH 6.5, but could be reversed by adjusting to pH 4.5, thus following the pH relationship for the precipitation of the BZ-copper complex. In similar experiments no inhibition of cytochrome oxidase activity could be obtained by incubating homogenates of normal plants with BZ at either pH.

Homogenates of BZ-modified plants were next subjected to various treatments in attempts to increase their copper-enzyme activity. Overnight incubation with pH 4.5 buffer was ineffective, suggesting that the low activity was not due to direct complexing of copper in the enzymes by BZ. Dialysis overnight against pH 4.5 or 6.5 buffers containing 10^{-7} to 10^{-4} M copper sulfate also proved unsuccessful, eliminating mere lack of exchangeable copper as the factor limiting activity.

Copper Content of Normal and BZ-modified Plants: To see whether the reduced copper-enzyme activity of modified plants was due to actual copper deficiency, copper determinations were carried out by the method of Eden and Green (11), on two 500 mg dry samples of each type. The results showed a copper content of about 16 μg/gm on a dry weight basis for the normal plants and about 22 μg/gm for the modified plants. Both these values gave a fresh-weight copper content of 0.88 to 0.99 μg/gm, and thus did not reflect the changed enzymatic activity.

Trace Elements and the Morphological Effects of BZ; Other Complexing Agents: Increased amounts of Cu, Zn, Fe, or Mn added to the medium, singly or in combinations, up to 64 times the usual concentrations, would not prevent the morphological effects of BZ. BZ did not affect the toxicity of the higher concentrations. The action of BZ was not changed by cobalt chloride or Vitamin B_{12} over a wide range of concentrations.

Attempts were made to imitate the morphological effects of BZ by growing plants with a wide range of concentrations of compounds known to complex copper or the other metals complexed by BZ; all proved unsuccessful. The compounds were: salicylaldoxime, alphabenzoinoxime, diphenylthiocarbazone, diphenyl carbazide, phenylthiohydantoic acid (1), PTU, and ethylenediaminetetraacetic acid. 5,6-dimethylbenzimidazole did not imitate the action of BZ.

Discussion

Two reports of the use of BZ on higher plants have appeared. Moore (21) tested it as a growth inhibitor, but made no attempt to determine its mode of action. Galston et al (13) found that 1 to 3×10^{-3} M in the presence of high auxin concentrations inhibited the auxin-induced elongation of etiolated pea epicotyl sections while enhancing transverse cell enlargement and water intake. The fact that they did not observe an effect of BZ in the absence of auxin led them to suggest that it specifically affects auxin action. Adenine, adenosine and other purines tried caused at most a 20 % reversal of the effects of BZ.

The action of BZ as an antipurine was originally reported by Woolley (32). Subsequent work has not confirmed his results, and evidence on the question, as reviewed by Wright (33), is inconclusive. More recently, Hughes (17) failed to reverse the action of BZ on chick cell tissue with any purine tried. Similar negative results were obtained in the present investigation.

The action of BZ on Lemna has at least two distinct aspects, which may or may not be related. Morphologically, BZ caused almost total inhibition of root elongation, 65 to 75 % frond enlargement due to increased cell size (table I), and marked frond epinasty. Biochemically, several differences were observed between normal and BZ-modified plants. The oxygen consumption of modified plants was unaffected or increased by concentrations of metal-enzyme inhibitors (Cn, N_3, CO, PTU) which inhibited that of the normal plants. No such differential effect was given by 2,4-dinitrophenol or iodoacetate, which affect

other than metal enzymes (tables II, III). Enzyme assays (table IV) of homogenates showed a reduction (compared to the normal) of all activities in modified plants, but the virtual absence of polyphenol oxidase and ascorbic acid oxidation called attention to a possible relation between BZ and copper.

BZ forms complexes with copper, zinc, cobalt, cadmium and nickel, but not with iron (29). Overnight storage with BZ solutions inhibited the copper enzyme activity of homogenates of normal plants, but not the cytochrome oxidase activity. The reversal of this direct inhibition by low pH paralleled the effect of low pH in reducing the morphological effects of BZ and in preventing the precipitation of the BZ-copper complex. These facts suggest that BZ acts by complexing copper, but no less copper was found in the modified plants than in the normal, and the symptoms reported for copper deficiency in Lemna (30) do not resemble the BZ-induced modifications. Most probably, then, BZ sequesters copper within the plant, inhibiting some but not all of the processes requiring the metal. The inability of additional copper to increase copper-enzyme activity when added to homogenates of BZ-modified plants argues for the absence of the necessary apoenzymes in such homogenates, but more evidence would be required to confirm this hypothesis. The marked reduction in cytochrome oxidase activity in homogenates of modified plants may be explained by the work of Elvehjem (12) and others (9, 24, 25) showing that cytochrome synthesis and cytochrome oxidase activity in diverse organisms is closely correlated with copper nutrition. In a similar fashion, copper might be necessary for the continued synthesis of the protein moieties of the copper containing enzymes.

While it is clear that a disturbance in copper metabolism is thus induced by the copper complexing properties of BZ, there is no evidence that the morphological effects of the compound are due to this action. The inability of increased copper or other trace metals to prevent these effects, and of other complexing agents to duplicate them, weighs against this possibility, although without eliminating it. Since the increase in frond area induced by BZ was due to cell enlargement it resembled one of the effects observed by Galston et al (13). Certain levels of auxins caused morphological changes in Lemna similar to those induced by BZ, while normal and BZ-modified plants showed different sensitivities to auxins and an antiauxin (fig 2). These observations provide additional evidence that BZ affects auxin metabolism, although the mechanism remains unknown. It is probably this aspect of BZ action which underlies the morphological changes caused by the compound. In view of recent work relating auxin-induced cell elongation to ascorbic acid oxidase activity (22, 23) it is possible that the disturbance in copper metabolism is somehow the cause of the change in auxin relations. Hackett et al (16), however, have found no evidence for a role of copper-enzymes in auxin-induced growth.

The differential effect of metal enzyme inhibitors on the oxygen consumption of normal and modified plants must reflect some difference in their terminal oxidase systems. Since all the metal enzyme inhibitors reduced the oxygen consumption of the normal plants, at least part of this must be mediated by metal-containing systems. PTU was the least effective inhibitor, suggesting that while a copper system may be operative it is not the only metal containing system present. Cytochrome oxidase is probably the non-copper metal system active (18), but the copper system and the apparent residual non-metal system cannot be further identified by these data. The inhibitor results are thus partially explained by the effects of BZ in reducing both copper enzyme and cytochrome oxidase activity. However, the increase in oxygen consumption caused in the modified plants by at least two of the inhibitors in question also seems to imply the existence of an oxidase system which is stimulated by metal-complexing agents, and cannot be satisfactorily explained at present.

Summary

Benzimidazole (BZ) supplied to the medium of aseptically cultured Lemna at about 3.4×10^{-3} M induces a modification characterized by a complete inhibition of root elongation and a 65 to 75 % increase in frond area due to cell enlargement. High auxin concentrations induce a similar modification in Lemna but plants so produced soon die, in contrast with the continued viability of the BZ-modified plants. BZ-modified plants exhibit a higher sensitivity to auxins and a lower sensitivity to the antiauxin 2,4,6-T than do normal plants. Attemps to prevent the BZ-modification with adenine and related compounds proved unsuccessful.

Oxygen consumption of modified plants is unaffected, or increased, by concentrations of cyanide, azide, carbon monoxide and phenylthiourea which inhibit that of normal plants. Homogenates of modified plants have 0 to 4 % of the polyphenol oxidase and ascorbic acid oxidation activity, 20 to 33 % of the cytochrome oxidase activity, and 45 % or more of the catalase and glycolic acid oxidase activity of normal homogenates, on a fresh weight basis. BZ is a weak direct inhibitor of polyphenol oxidase activity, but not of cytochrome oxidase, in normal homogenates. It forms an insoluble complex with copper. Modified plants contain no less copper than the normal. It is concluded that at least some of the effects of BZ are due to its sequestration of copper within the plant and to the resultant disturbance in copper metabolism.

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Literature Cited

1. Albert, A. and Gledhill, W. S. The choice of a chelating agent for inactivating trace metals. Biochem. Jour. 41: 529–545. 1947.

2. Altschul, A. M., Karon, M. L., and Kyame, L. Catalase and peroxidase in germinating cotton seed. Arch. Biochem. 18: 161–170. 1948.
3. Ashby, E., Wangermann, E., and Winter, E. J. Studies in the morphogenesis of leaves. III. Preliminary observations on vegetative growth in *Lemna minor*. New Phytologist 48: 374–381. 1949.
4. Bonner, D. M. and Haagen-Smit, A. J. Leaf growth factors. II. The activity of pure substances in leaf growth. Proc. Nat. Acad. Sci, U. S. 25: 184–188. 1939.
5. Bonner, J. and Bandurski, R. S. Studies of the physiology, pharmacology, and biochemistry of the auxins. Ann. Rev. Plant Physiol. 3: 59–86. 1952.
6. Brown, R. and Rickless, P. A new method for the study of cell division and cell extension with preliminary observations on the effect of temperature and nutrients. Proc. Roy. Soc. (London) B 156: 110–125. 1949.
7. Burström, H. Physiology of root growth. Ann. Rev. Plant Physiol. 4: 237–252. 1953.
8. Clagett, C. O., Tolbert, N. E., and Burris, R. H. Oxidation of alphahydroxy acids by enzymes from plants. Jour. Biol. Chem. 178: 977–987. 1949.
9. Cohen, E. and Elvehjem, C. A. The relation of iron and copper to the cytochrome and oxidase content of animal tissues. Jour. Biol. Chem. 107: 97–105. 1934.
10. DuBois, K. P. and Erway, W. F. Studies on the mechanism of action of thiourea and related compounds. II. Inhibition of oxidative enzymes and oxidations catalyzed by copper. Jour. Biol. Chem. 165: 711–721. 1946.
11. Eden, A. and Green, H. H. Microdetermination of copper in biological materials. Biochem. Jour. 34: 1202–1208. 1940.
12. Elvehjem, C. A. The role of iron and copper in the growth and metabolism of yeast. Jour. Biol. Chem. 90: 111–132. 1930.
13. Galston, A. W., Baker, R. S., and King, J. W. Benzimidazole and the geometry of cell growth. Physiol. Plantarum 6: 863–972. 1953.
14. Goddard, D. R. and Holden, C. Cytochrome oxidase in the potato tuber. Arch. Biochem. 27: 41–47. 1950.
15. Gorham, P. R. Heterotrophic nutrition of seed plants with particular reference to *Lemna minor* L. Canadian Jour. Research C 28: 356–381. 1950.
16. Hackett, D. P. and Schneidermann, H. A. Terminal oxidases and growth in plant tissues. I, II (with Thimann, K. V.). Arch. Biochem. Biophys. 47: 190–214. 1953.
17. Hughes, A. The effect of purines and related substances upon cells in chick tissue. Exptl. Cell Research 3: 108–120. 1952.
18. James, W. O. Plant Respiration. Pp. 1–282. Clarendon Press, Oxford. 1953.
19. McRae, D. H. and Bonner, J. Diortho substituted phenoxyacetic acids as antiauxins. Plant Physiol. 27: 834–838. 1952.
20. Millerd, A., Bonner, J., Axelrod, B., and Bandurski, R. S. Oxidative and phosphorylative activity of plant mitochondria. Proc. Nat. Acad. Sci, U. S. 37: 855–862. 1951.
21. Moore, A. M. Growth Inhibition studies. I. Measurement of the effects of inhibitors on growth rate of flax seedlings. Jour. Exptl. Bot. 4: 23–31. 1953.
22. Newcomb, E. H. Effect of auxin on ascorbic oxidase activity in tobacco pith cells. Proc. Soc. Exptl. Biol. Med. 76: 504–509. 1951.
23. Newcomb, E. H. Separation of metabolic effects of auxin by phenylthiourea. Abstract, Program of Meetings, Amer. Soc. Plant Physiol., Madison, Wisconsin. September, 1953.
24. Schultze, M. O. The effect of deficiencies in copper and iron on the cytochrome oxidase of rat tissues. Jour. Biol. Chem. 129: 729–737. 1939.
25. Schultze, M. O. The relation of copper to cytochrome oxidase and hematopoietic activity of the bone marrow of rats. Jour. Biol. Chem. 138: 219–224. 1941.
26. Shive, W. Biological activities of metabolite analogues. Ann. Rev. Microbiol. 6: 437–466. 1952.
27. Simon, E. W. and Beevers, H. The effect of pH on the biological activities of weak acids and bases. I. The most usual relationship between pH and activity. New Phytologist 51: 164–190. 1952.
28. Skoog, F. and Tsui, C. Chemical control of growth and bud formation in tobacco stem segments cultured in vitro. Amer. Jour. Bot. 35: 782–787. 1948.
29. Skraup, S. Additionsreaktionen und Ringspaltungen einiger heterocyclischen Verbindungen. Annalen Chemie, Justus Liebigs 419: 1–92 (see 70–71). 1919.
30. Steinberg, R. A. Mineral requirements of *Lemna minor*. Plant Physiol. 21: 42–48. 1946.
31. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques and Tissue Metabolism. Pp. 1–227. Burgess Publishing Co., Minneapolis, Minnesota. 1949.
32. Woolley, D. W. Some biological effects produced by benzimidazole and their reversal by purines. Jour. Biol. Chem. 152: 225–232. 1944.
33. Wright, L. D. Antimetabolites of nucleic acid metabolism. Vitamins and Hormones 9: 131–159. 1951.
34. Zimmerman, P. W. Formative effects of hormone-like growth regulators. In: Plant Growth Substances, F. Skoog, ed. Pp. 175–183. University of Wisconsin Press, Madison, Wisconsin. 1951.

CHANGES IN CATALASE ACTIVITY AND OTHER RESPONSES INDUCED IN PLANTS BY RED AND BLUE LIGHT[1]

DAVID APPLEMAN AND H. T. PYFROM
COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA, LOS ANGELES

In an earlier paper (1) it was reported that etiolated barley seedlings when exposed to visible radiation suffer a large decrease in catalase activity and a simultaneous increase in chlorophyll. Eyster (4) found that dark-grown genetic albino corn seedlings, completely devoid of chlorophyll, also suffer a loss in catalase activity when illuminated. There are several earlier studies such as Knott (7) that report the effects of light on the catalase activity of growing plants. In all these studies light of the whole visible spectrum was used. Since it is apparent that the decrease in catalase involves a photochemical reaction, it is reasonable to assume that certain regions of the spectrum may be more effective than others. We initially planned to determine the action spectrum for this process, but difficulties involved in obtaining light of relatively narrow spectral bands of sufficient intensity forced use to confine our studies for the present to red and blue. This paper reports the results of these studies.

MATERIALS AND METHODS

Barley, variety Atlas, was used in most of the experiments described in this paper. The seeds were soaked overnight, placed on stainless steel screens fitted into 400-ml beakers (fig 1), and kept in a saturated atmosphere in a dark room. Within six days the seedlings had attained a height of 4 to 6 cm; two lots were now removed from the dark chamber and placed in special chambers irradiated with red and blue light respectively. A descriptive sketch of the light chamber is given in figure 2. The sources of radiant energy were fluorescent tubes GE F14T12. The characteristics as given by the manufacturer are as follows: the red tubes do not emit any light shorter than 6000 Å and have a maximum emission at 6600 Å. The blue tubes have a maximum at 4400 Å and extend partially into the green to the region of about 5000 Å. Measurements were made of the irradiance as well as the total energy of the fluorescent tubes. In the center of the chamber eighteen red tubes gave a total irradiance of 180 fc and a total energy of 0.0308 cal/cm^2 × min (2.15×10^4 ergs/cm^2 × sec). Since 18 tubes are the maximum number that our chamber can accommodate, it was necessary to adjust the blue to the same total radiant energy by using a smaller number of tubes and/or colored glass filters. The temperature in the chambers was generally maintained at 24 ± 1° C unless otherwise stated.

After the plants were placed in the light chambers samples were periodically taken by cutting off the upper 2/3 of the first leaf blades. Weighed portions of these samples were used for determinations of dry weight, nitrogen, chlorophyll, and catalase. The methods used in these determinations are described elsewhere (1, 2). We define the catalase unit as the amount of catalase required to liberate from H_2O_2 one ml O_2/sec at 0° C, under our experimental conditions.

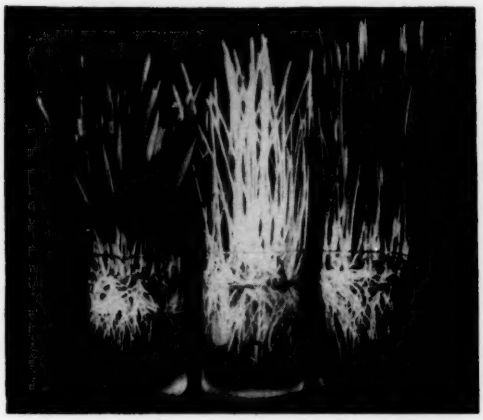

FIG. 1. Barley seedlings, showing the method of growing. From left to right plants grown in blue light, dark grown plants and plants grown in red light are 9 days from planting and 4 days from start of irradiation.

Several other plants were used to compare with barley, also some other determinations were made in addition to those mentioned. These are described in their proper places.

RESULTS

Typical effects of red and blue light are presented in figures 3 and 4. A comparison shows that percentage dry material and total nitrogen, calculated on a fresh weight basis, are approximately the same under both types of irradiation. In fact, the changes in both of these were very slight during the 8-day period that they were studied. The chlorophyll accumulates at approximately the same rate in red and blue grown plants during the first 2 days. After that there is no further accumulation in the plants grown in red light; however, the plants grown in blue light continue to accumulate chlorophyll until the 4th day, when a maximum about 33 % higher than that in red light is reached.

Catalase activity is high, initially, in the etiolated seedlings, and, as has been shown earlier (1) in the case of etiolated seedlings exposed to white light, there is an initial drop in activity. We find here that in plants grown in red light the decrease is small, roughly about 15 % and is reversed after 24 to 48 hours. In plants grown in blue light the decrease is large, about 70 %, and reaches a minimum activity in 4 to 5 days of irradiation. It should be noted that under both types of irradiation the minimum in catalase activity coincides with the most rapid chlorophyll synthesis,

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and catalase activity begins to increase again only after chlorophyll has reached its maximum.

The surprisingly high catalase activity of the red grown plants and the low value to which catalase drops in the blue grown plants prompted us to investigate whether these quantitative relationships could be reversed. An experiment was set up, therefore, to investigate this possibility.

Two lots of 5-day-old etiolated barley seedlings were placed in the chambers under the red and the blue light respectively. A third lot was continued in the dark. After four days several beakers of red

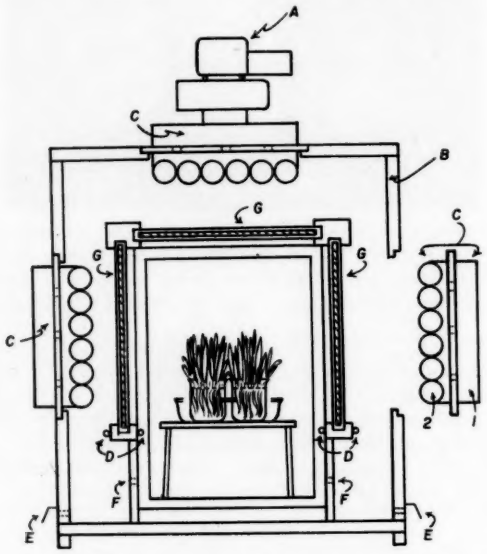

FIG. 2. Cross section of light chamber. Outside dimensions 24″ × 24″ × 25″ high. The space for plants is 14″ × 24″ × 18″ high. A—Exhaust fan, capacity 35 ft³/min. B—Walls of chamber made of ¾″ marine plywood. C—Light panels which are interchangeable (1—Light shield to prevent light from reaching slots in the mounting board, 2—Fluorescent tubes). D—Perforated ⅝″ copper tubes running the length of the chamber, infrequently used to blow in air. E—Air intake from the outside. F—Adjustable slot to permit the incoming air to go through the inner chamber. The outlets for this air are located in front and back of the frame holding the upper filter. G—¾″ slots for glass filter.

irradiated plants were placed in the dark and several in blue light. Also, plants which had been irradiated for four days in the blue chamber were likewise placed in red and in darkness respectively. The results of this experiment are shown in figure 5. It is apparent from the figure that the effect of light quality on catalase activity is rapidly reversible. Catalase activity is rapidly increased in red light and equally rapidly decreased by blue light. It is interesting that the blue plants placed in the dark, while increasing somewhat in catalase activity, did not reach the same high value as those placed in the red. This is contrary to the

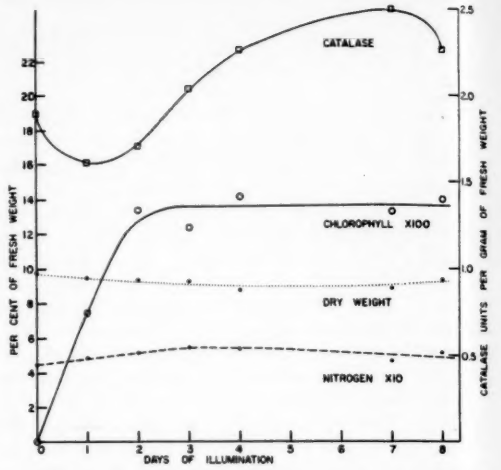

FIG. 3. Plants were grown for 5 days in dark room then placed in the red light chamber.

notion that plants grown in red light are similar to plants grown in the complete absence of light.

We next attempted to determine whether the previously described light effects were purely photochemical and therefore independent of temperature. By working in a room maintained at 0° C, we were able to keep the temperature inside the light chambers at 0° to 2° C. Plants kept in the dark were outside the chambers at a temperature that varied little from 0° C. Six-day-old etiolated seedlings were placed in the chambers and irradiated for four days. Catalase activity at this time was as follows: plants grown in dark 1.8 units/gm of fresh weight, plants grown in red light 2.0 units, plants grown in blue light 1.7 units. Comparable plants irradiated at 24° C for four days gave activities as follows: dark 2.2 units, red 2.3 units and blue 0.5 units. It is apparent that temperature does influence the light effects.

Talaricio (14) in 1909 reported that crude liver

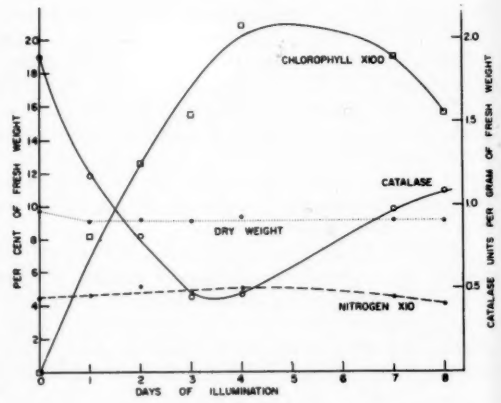

FIG. 4. Plant grown for 5 days in dark room and placed in blue light chamber.

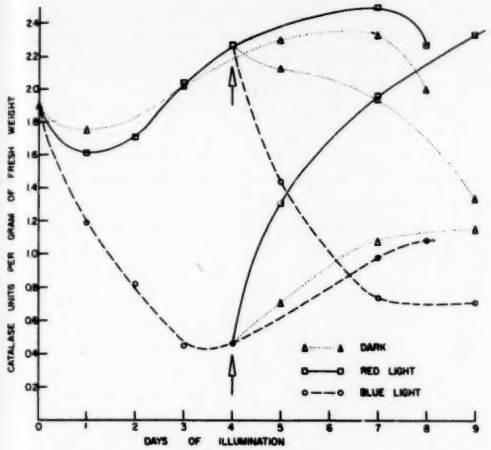

FIG. 5. Changes in catalase activity of plants grown in blue and red light when each is subjected to the other two light conditions. Thus blue grown plants are shifted to red and dark, and red grown plants are shifted to blue and dark. Transfer point is indicated by arrow.

catalase when exposed to blue light suffered a loss in activity, while when exposed to red light it either remained constant or showed a slight increase in activity. Murakami (8) studied the effect of monochromatic light on a number of enzymes, including catalase, in autolyzed yeast and found an increase in activity of catalase in the blue light greater than in red light. We thought, therefore, it was desirable to check the effect of the light on the tissue homogenates. We exposed homogenates of barley leaves and also of avocado fruit tissue, which is very high in catalase, to red and blue light for periods varying from one hour to four days. No change in the catalase activity was produced as compared to samples kept for the same period of time in complete darkness. Since our experimental results do not agree with those of Talaricio and Murakami, we must conclude that our observed photochemical reactions required organization of the cell and are probably part of a complex system in which other living processes are involved.

This consideration prompted us to investigate other physiological and biochemical changes that occur in plants when they are irradiated with red and blue light. We do not wish to imply that the changes reported below are necessarily, either directly or indirectly, related to the changes in catalase activity. The values for catalase given along with the other data are simply to indicate that the irradiation was effective in producing some change.

NITROGEN: There are some inferences, in the literature dealing with morphological effects of irradiation, that the blue end of the spectrum accelerates protein synthesis in plants. Voskresenskaya (15) in a recent paper concludes that the blue-violet end of the spectrum favors protein synthesis, while the longer wave lengths favor the synthesis of carbohydrates. In this

TABLE I

DISTRIBUTION OF PROTEIN, AMINO ACID AND OTHER NITROGEN FRACTIONS IN LEAVES OF 10-DAY-OLD BARLEY SEEDLINGS WHICH FOR THE LAST 4 DAYS WERE EXPOSED TO BLUE OR RED LIGHT

NITROGEN FRACTION	BLUE	RED	DARK CONTROL
	mg N/gm fresh wt		
Amino acids and amides ..	0.43	0.54	1.07
Polypeptides	0.19	0.19	0.14
Proteins	2.90	2.62	2.81
Total N	3.52	3.35	4.02
Catalase units/gm fresh wt	1.08	2.22	2.27

it was of interest to see whether a relatively short period of irradiation with red or blue light produces a detectable effect on the protein content of barley seedlings.

Representative samples of 10-day-old barley leaves which had been exposed for 4 days to either red or blue light were subjected to a nitrogen fractionation by the method of Bathurst (3). The results given in table I indicate that the blue irradiated leaves have a smaller amount of free amino acids than the red irradiated leaves but a larger amount of protein. The higher total nitrogen in the dark grown leaves does not indicate a higher nitrogen per leaf but a lower fresh weight per leaf. It appears that red light makes for greater growth in length, and blue light for greater protein synthesis. Admittedly this study by itself is inadequate to establish this observation. However, this view gains support from many total nitrogen determinations (Kjeldahl) on a per plant basis which show that blue irradiated leaves have a consistently higher total nitrogen than leaves of the same age irradiated with red light or grown in the dark. The data on pea epicotyls given in table VI lend additional support.

ASCORBIC ACID: It is well known that reducing substances such as glutathione, cystein and ascorbic acid have an inhibitory effect on catalase activity in vitro (5). It is also known that accumulation of ascorbic acid in seedlings is affected by light (11). It was thought desirable to determine whether there are differences in ascorbic acid in red and blue irradiated seedlings. Ascorbic acid (AA) was determined by the method of Ramsey and Colichman (10). The ascorbic plus the dehydroascorbic acid was determined by the

TABLE II

ASCORBIC ACID (AA) AND DEHYDROASCORBIC ACID (DAA) CONTENT OF BARLEY LEAVES FROM 10-DAY-OLD PLANTS IRRADIATED WITH BLUE AND RED LIGHT AND DARK GROWN

LIGHT	ASCORBIC ACID (MG/GM FRESH WT)		CATALASE UNITS/MG N	CATALASE UNITS/GM FRESH WT
	AA	AA + DAA		
Blue	0.19	0.27	0.27	1.2
Red	0.06	0.11	0.61	2.2
Dark	0.10	0.17	0.49	2.2

same method on an aliquot of leaf homogenate through which H_2S had been bubbled. The results of a typical determination are given in table II. There is definitely a higher ascorbic acid content in blue irradiated leaves as compared to either dark or red. However, adding varying concentrations of AA to the culture solution of red irradiated seedlings or spraying the leaves with dilute solutions of AA did not influence the catalase activity of the leaves.

HILL REACTION: The photolysis of water by isolated chloroplasts is known to be cyanide insensitive. It, therefore, has been assumed that catalase does not directly participate in it. There remained, however, the possibility that the chloroplasts of plants irradiated with red and blue light may have other changes produced in them which do affect their Hill reaction capacity.

The method we used for both chloroplast preparation and measurement of their capacity for photoreduction was essentially that of Spikes (13). The Hill reagent used was 0.005 M potassium ferricyanide. Red light of approximately 1500 fc was supplied from a 300-watt reflector spot lamp transmitted through a double thickness of red cellophane. The reaction temperature was maintained at 10° C and a Beckman model G pH meter was used as a potentiometer.

Admittedly our setup was crude, compared to the very elegant apparatus of Spikes. The results, however, were consistent and reproducible. Typical Hill reaction results are summarized in table III. In all the plants studied the chloroplast materials from red irradiated plants are only 60 to 70 % as efficient in photoreduction of ferricyanide as are their blue counterparts. The following additional information on the plant material used in Hill reaction studies given in table III should be noted. The barley plants varied in age from 8 to 14 days and had been exposed to red and blue light for 4 days. The corn was a dwarf variety 10 days from seed when used and had had 4 days of red or blue irradiation. The tobacco plants were grown in soil in a glasshouse for about 6 weeks, and subsequently irradiated in the light chambers for 6 days prior to their use; only the young leaves which had expanded in the light chamber were used. Experiment 5, table III was performed as follows: Two sets of 6-day-old plants were placed in each of the two chambers. After 4 days one set from each chamber was removed and placed in the other chamber. Thus B→R designates a plant that was in the blue chamber 4 days and was then shifted to the red chamber and similarly for R→B. After another 4 days the plants were harvested and used for Hill effect determinations. It appears that the exposure of a plant to blue light produces an irreversible effect with respect to the Hill reaction. This effect is not changed by exposing the plant to red light either prior or subsequent to its exposure to blue light. The catalase activity values given in this table are just to indicate the degree to which the plant has reacted to the irradiation.

EXPERIMENTS ON BARLEY COLEOPTILES: So far we have considered the effect of red and blue light on leaves. Because of the general interest in coleoptiles as a light receptive tissue, it was thought desirable to determine how their catalase activity is affected by similar irradiation.

Barley seedlings were grown in the dark room as previously described. Four to six days after planting, between 50 and 100 coleoptiles were detached from the seedlings in the dark and placed in light-proof aluminum weighing cans, quickly weighed and transferred to Petri dishes on moist filter paper. The coleoptiles, in the covered dishes, were placed in the red and blue light chambers or in darkness for predetermined periods of time. The coleoptiles from each dish were homogenized and the various determinations made on the homogenate. In a number of experiments the intact seedlings were irradiated and the coleoptiles detached after the period of irradiation. These are referred to in table IV as "attached." It is apparent from the results that catalase activity in the

TABLE III

HILL REACTION DETERMINATIONS ON CHLOROPLASTS FROM COMPARABLE BLUE AND RED IRRADIATED PLANTS

EXPT. NO.	PLANT AND COLOR OF LIGHT	CHLOROPHYLL IN SAMPLE, MG	MICROEQUIVALENTS OF Fe^{+++} REDUCED/MG CHLOROPHYLL PER MINUTE	CATALASE UNITS IN PLASTID SUSP/MG CHLOROPHYLL	CATALASE UNITS IN LEAF/MG N	RELATIVE HILL REACTION R/B
1	Barley B	1.75	0.974	0.124	...	0.59
	Barley R	1.82	0.577	0.938	...	...
2	Barley B	1.60	0.938	0.168	...	0.64
	Barley R	1.98	0.606	0.766	...	...
3	Corn B	2.66	0.624	...	0.09	...
	Corn R	2.36	0.435	...	0.18	0.69
4	Tobacco B	1.25	1.40	...	0.23	...
	Tobacco R	0.48	0.85	...	0.66	0.60
5	Barley B	2.60	1.44	0.18	0.35	...
	Barley R	2.00	0.98	0.63	0.72	0.68
	Barley B → R	2.22	1.39	0.58	0.64	...
	Barley R → B	2.58	1.34	0.21	0.43	...

TABLE IV

EFFECT OF FOUR HOURS OF COLORED LIGHT ON CATALASE ACTIVITY IN BARLEY COLEOPTILES (AVERAGE OF FOUR EXPERIMENTS)

TREATMENT	CATALASE UNITS/GM N	
	ATTACHED	DETACHED
Dark control	404	449
Red light	384	455
Blue light	234	223

dark grown coleoptiles is reduced to approximately half when exposed to blue light for four hours but is very slightly influenced by exposure to red light. The data in table IV are average results of four experiments in our regular light chambers. In addition we performed many experiments with detached coleoptiles using high intensity incandescent lights and Corning glass filters to obtain the desired light intensity but a higher degree of spectral purity. The primary purpose of these experiments was to ascertain whether the effect of blue light on catalase was not due to the small amount of "far-red" (7400 Å) which the blue fluorescent tubes emit. To eliminate the far-red, blue filter No. 42 from a Klett-Summerson photo-colorimeter was used. It transmits very little light of the far-red region. To obtain the far-red, Corning filter No. 5874, which transmits the far-red and also a band in the violet, was used. The results of these experiments have satisfactorily shown that the observed effect on catalase by the use of blue fluorescent tubes is due to the blue light and not to the small amounts of far-red.

It is worth pointing out the advantages of using the barley coleoptile for this type of research. The complication of the presence or formation of chlorophyll is eliminated, since the quantity involved is insignificant. The time required to produce a change in catalase is relatively short. Also, the experimental manipulation is much simpler than with whole seedlings.

EFFECTS OF RED AND BLUE LIGHT ON PLANTS OTHER THAN BARLEY: For most of our work barley seedlings were the experimental material. We have, however, made many determinations on other plants. Some of these we feel are worth recording for the information of those who may wish to work with other plants. In general, all the grain seedlings we used (oats, corn, wheat) behave like the barley. With respect to the dicotyledonous plants, table V shows catalase data on several species from two families. Since sedlings of dicotyledonous plants do not form leaves when etiolated, the plants were grown in a glasshouse until they developed several normal mature leaves. They were then placed in the light chambers for 3 to 6 days and catalase determinations were subsequently made on the leaves. It is evident that the catalase activity in the two species of Solanaceae is affected by the monochromatic light in the same way as the grains. The Leguminosae, on the other hand, react in the opposite manner.

TABLE V

EFFECT OF RED AND BLUE LIGHT ON LEAF CATALASE OF SEVERAL PLANTS OTHER THAN BARLEY

PLANT	WEEKS OF DAYLIGHT IN GLASSHOUSE	DAYS IN LIGHT CHAMBER	CATALASE UNITS/GM N	
			RED	BLUE
Tobacco	6	6	660	230
Tomato	3	4	550	325
Bean	4	4	230	385
Clover	6	4	554	688
Pea	4	4	325	405

In view of these observations, we set up an experiment on pea seedlings. Alaska peas were soaked for 4 hours, then planted in moist vermiculite in 400-ml beakers. The seedlings were left to grow in complete darkness for four days. By this time they were 5 to 6 cm high. Part of them were now removed from the dark room and placed in the red and blue chambers. After 2 days of irradiation the seedlings were cut at the surface of the vermiculite and subjected to the regular analyses for catalase and nitrogen. Subsequently other samples were taken after 4 days' irradiation. The results given in table VI indicate some striking differences from the barley seedlings. The catalase activity and the total nitrogen in the dark grown seedlings are very much lower than in either the red or the blue irradiated seedlings. Also the catalase activity differences between plants grown in red and blue are much smaller and appear to be decreasing with continued irradiation and age. Actually the leaves of the young plants exposed to blue light have a higher catalase activity than those exposed to red. It seems evident that the catalase picture in pea epicotyls is more complex and subject to more rapid changes than that of barley. The whole subject of catalase in legumes shows promise of an interesting study.

TABLE VI

EFFECT OF RED AND BLUE LIGHT ON CATALASE ACTIVITY IN PEA EPICOTYLS

TREATMENT	MG N/GM FRESH WT	CATALASE UNITS/GM FRESH WT	CATALASE UNITS/GM N
Dark 6 days	5.04	0.76	150
Dark 4 Red 2	8.54	2.70	304
Dark 4 Blue 2 ...	8.90	2.07	242
Dark 8 days	4.88	0.43	87
Dark 4 Red 4	9.20	2.42	263
Dark 4 Blue 4 ...	10.40	2.38	229

DISCUSSION

From the experimental results presented it is clear that the loss of catalase activity in etiolated barley seedlings, when exposed to visible light, is due to the light of the blue end of the spectrum. Also this decrease in activity may be rapidly restored by irradia-

tion with light of the red end of the spectrum. Neither the reduction of activity by blue light nor the restoration, or actual increase, in activity by red light takes place in the tissue homogenate. This indicates that grinding the tissue produces changes either in the receptor pigment or in the thermal components of the system. At present no attempt has been made to identify the receptor pigment. It certainly is not chlorophyll, since non-chlorophyllous tissues such as barley coleoptiles and *Prototheca zopfii* (author's unpublished work) are equally affected. It may possibly be catalase or some other heme compound. We do not think it likely that leaves of leguminous plants have a different receptor pigment than the other plants studied. Their different reaction to red and blue light we are inclined to ascribe to the thermal rather than the photochemical phase of the reaction.

The other effects of red and blue light, such as the effect on the Hill reaction, indicate that other enzymes may also be changed by light from these regions of the spectrum. This presents an interesting experimental approach to other plant physiological problems.

The initial drop in catalase on irradiation of etiolated barley seedlings with light of any part of the spectrum, we think, is significant. The initial decrease of catalase in the red grown plants is relatively small. We have, therefore, checked it many times, and we are convinced that the decrease is indeed real. The maximum drop in catalase under all light conditions used coincides with the period of most active chlorophyll synthesis. This is in agreement with other instances known where active synthetic activity in an organism or tissue is accomplished or preceded by a decrease in catalase activity. It is well known, for example, that the liver and kidney catalase of a tumor host is greatly reduced during the period of most rapid tumor growth and is very rapidly restored on excision of the tumor. Similarly, there is a drop in catalase activity in rats during pregnancy. Also, the embryonic liver of rats is very much lower in catalase than the adult liver. Pope (9) studied catalase in barley throughout the life cycle for three successive years. He concludes, "Catalase activities are roughly proportional to the reciprocal of growth rate, being, in general, lowest during the stages of most active growth, as measured by length and deposition of dry matter." Unfortunately most of the work on catalase in plants that one finds in the literature is not accompanied by sufficient data on the physiological state of the tissue or organism to permit further deductions.

It is not known, at present, what actually happens to the catalase in the living cell, when a determination of catalase activity on the cell homogenate indicates a decrease. Does it represent a major breakdown of the whole molecule or a small, reversible change, perhaps, concerned with the linkage of the prosthetic group to the protein? Such information would be very helpful in explaining some of our observations. But, whatever the change in activity may be due to, it is reasonable to assume that a change in catalase activity is reflected in an opposite change in the H_2O_2 concentration in the cell. The hydrogen peroxide, because of its capacity to initiate free radical formation, may play an important role in biosynthetic processes. In this regard our data are in harmony with the speculations of Rondoni and Cudkowicz (12) on the indirect function of catalase in protein synthesis. Another aspect of growth over which catalase may have an indirect control, is illustrated in the scheme for indoleacetic acid oxidase as proposed by Galston and coworkers (6). We appreciate the fact that catalase controls only the decomposition of H_2O_2 and that factors involved in the formation of peroxide must also be taken into consideration. We simply wish to suggest that catalase, because of its control on H_2O_2 concentration in the cell, may be exerting a powerful control over several physiological processes.

Summary

Changes in catalase activity in plants, caused by irradiation for short periods of time with red or blue light, were studied. It was found that:

1. In etiolated barley seedlings and other grains, catalase activity is greatly depressed by blue light and elevated by red light. This is also true for young tomato and tobacco plants grown in normal daylight and illuminated with colored light for 3 to 6 days.

2. Catalase in legumes (beans, peas and clover) was increased by irradiation with blue light and decreased by red light.

3. Quantitative changes, due to red or blue light, in the Hill reaction and in ascorbic acid and protein content are reported.

4. The probable significance of catalase in biosynthetic processes is discussed.

Literature Cited

1. Appleman, David. Catalase-chlorophyll relationship in barley seedlings. Plant Physiol. 27: 613–621. 1952.
2. Appleman, David. Manometric determination of catalase activity: Apparatus and method. Anal. Chem. 23: 1627–1632. 1951.
3. Bathurst, N. O. Free amino acids and peptides of plant tissues. Jour. Sci. Food Agr. 4: 221–226. 1953.
4. Eyster, H. C. Catalase activity in chloroplast pigment deficient types of corn. Plant Physiol. 25: 630–638. 1950.
5. Foulkes, E. C. and Lemberg, R. The inhibition of catalase by ascorbic acid. Australian Jour. Exptl. Med. Sci. 26: 307–313. 1948.
6. Galston, A. W., Bonner, James, and Baker, R. S. Flavoprotein and peroxidase as components of the indole acetic acid oxidase system of peas. Arch. Biochem. Biophys. 42: 456–470. 1953.
7. Knott, James E. Rapidity of response of spinach to changes in photoperiod. Plant Physiol. 7: 125–130. 1932.
8. Murakami, Reitero. Influence of monochromatic light on the action of enzymes. Jour. Agr. Chem. Soc., Japan 16: 55–68. 1940.
9. Pope, M. N. Catalase activity in relation to the growth curve in barley. Jour. Agr. Research 44: 343–355. 1932.

10. Ramsey, J. B. and Colichman, E. L. The potentiometric determination of vitamin C. Ind. Eng. Chem., Anal. Ed. 14: 319–321. 1942.
11. Robertson, T. S. B. The accumulation of vitamin C during germination of peas. Australian Jour. Exptl. Biol. Med. Sci. 25: 41–46. 1947.
12. Rondoni, P. and Cudkowicz, G. Hydrogen peroxide in tumors. Experientia 9: 348–353. 1953.
13. Spikes, John D. Stoichemistry of the photolysis of water by illuminated chloroplast fragments. Arch. Biochem. Biophys. 35: 101–109. 1952.
14. Talaricio, G. Comportamento della catalisi del fegato alle luci monochromatische. Arch. Pharmacol. Sper. 8: 81–109. 1909.
15. Voskresenskaya, N. P. The effect of conditions of illumination on the composition of the products of photosynthesis. Trudy Inst. Fiziol. Rastenii, im. K. A. Timiryazeva, Akad. Nauk S.S.S.R. 8: 1 (1953): 42–56. 1953.

EFFECTIVENESS OF ETHYLENE DIAMINE TETRA-ACETIC ACID IN THE ACTIVATION OF OXIDATIONS MEDIATED BY MITOCHONDRIA FROM BROCCOLI BUDS[1]

M. LIEBERMAN[2] AND J. B. BIALE

Biological Sciences Branch, Agricultural Marketing Service,
U. S. Department of Agriculture and Department of Subtropical Horticulture,
University of California, Los Angeles, California

Mitochondria prepared from broccoli buds by the usual procedure, using 0.5 M sucrose as the homogenizing medium, showed poor activity. The rate of oxidation of succinate rapidly declined with time and there was virtually no oxidation of α-ketoglutarate. Several workers, with both plant (10, 16) and animal (3, 14) material, reported that disodium EDTA (disodium ethylene-diamine tetra-acetic acid) added to the preparative medium activated and stabilized mitochondrial oxidations. This type of activation was obtained with mitochondria from broccoli buds. The object of the present study was to determine the effectiveness of EDTA on broccoli mitochondria and to obtain clues as to the mechanism of activation.

Materials and Methods

With some modifications the method outlined by Laties (9) was used to prepare the mitochondria. In the preparative procedures all manipulations were carried out at 0° C. The broccoli buds were cut off the flower stalks and 12 gm were ground in a mortar with 20 gm of fine acid-washed sand and 25 ml of either 0.5 M sucrose or 0.5 M sucrose containing 0.01 M EDTA. Additional 10-ml portions of preparative medium were added to aid in homogenizing the tissue. Finally the homogenate was diluted with the same medium to make a total volume of 90 ml containing the 12 gm of tissue.

The resulting homogenate was squeezed through four layers of cheesecloth and the filtrate centrifuged at $1000 \times g$ for 5 minutes to precipitate pollen grains, unbroken cells, nuclei, cell debris and sand. The supernatant was then centrifuged at $17{,}000 \times g$ for 15 minutes. The second precipitate, which yielded the mitochondrial pellet, is described as "once washed".

A "twice washed" preparation was one in which the pellet was resuspended in 20 ml of 0.5 M sucrose and homogenized in the centrifuge tube with a snug-fitting lucite pestle. The resuspended particles were spun down at $17{,}000 \times g$ for 15 minutes. This procedure was repeated once more to give a "thrice washed" preparation. After all washes the final mitochondrial pellet was suspended in 4 ml of 0.5 M sucrose and homogenized with a motor-driven Teflon pestle.

The oxidative capacity of the mitochondria was determined by their ability to oxidize α-ketoglutarate or succinate. This was assayed at 25° C by standard Warburg manometric techniques. Details as to the content of the reaction mixtures are given in the legend for each figure.

Each experiment was replicated at least 3 or 4 times and the data are averages of these experiments. The data are reported in microliters oxygen per milligram nitrogen per hour designated as $Q_{O_2}(N)$.

Results

Activation and Stabilization Effect of EDTA: The effect of isolating mitochondria in 0.5 M sucrose containing 0.01 M disodium EDTA and in 0.5 M sucrose is shown in figures 1 and 2. With α-ketoglutarate as substrate the preparation without EDTA showed very low activity ($Q_{O_2}(N)$ of approximately 40). The preparation with EDTA exhibited a fourfold increase in oxidation ($Q_{O_2}(N)$ of approximately 160), which was maintained for at least 2 hours. A similar activation was shown with succinate as a substrate (fig 2). The most striking effect, especially with succinate, was the stabilization of activity for at least a 2-hour period.

Effect of Washing on the Activation Effect of EDTA: The purpose of the washing experiments was to reveal whether the activation affected by preparation in EDTA could be reversed by removal of the EDTA. In these experiments the activity of EDTA-prepared mitochondria washed once was compared with preparations washed twice and thrice with 0.5 M sucrose. Removal of EDTA by washing did not

[1] Received June 24, 1955.
[2] Present Address: Quality Maintenance Section, Plant Industry Station, Beltsville, Maryland.

reverse the activation effect (fig 3). The EDTA in the mitochondria and the supernatant before and after washing was determined by the method of Darbey (5). In an aliquot calculated to have 3.7 mg of EDTA the first washing contained 3.5 mg and no EDTA was retained in the mitochondria. These data indicated that all the EDTA was washed out of the mitochondria in the first washing.

From these data it appears that once the mitochondria are prepared in EDTA they cannot be inactivated by removal of the EDTA. It is also evident that the EDTA is completely removed by washing. The activation can, therefore, be interpreted as due to a chelation effect which does not require the presence of EDTA after sequestering has occurred.

Comparison of an Active and Inactive Form of EDTA: In order to determine whether the EDTA molecule as such is effective regardless of the nature of the attached cation, the action of ferric EDTA was compared with that of disodium EDTA. Disodium EDTA has a complex formation constant (K) of approximately 1.6, while ferric EDTA has a K of 25.1 (11, 12). Consequently ferric EDTA is very feebly dissociated and cannot be displaced by any of the common cations (11, 12). For all practical purposes ferric EDTA could not be considered a chelating agent of any of the common cations found in a cell. It was therefore interesting to determine its effect on the mitochondria as compared with that of disodium EDTA.

The results of these experiments are given in figure 4. These data clearly show that ferric EDTA does not activate the mitochondria, while disodium EDTA markedly activates them. Since both sub-

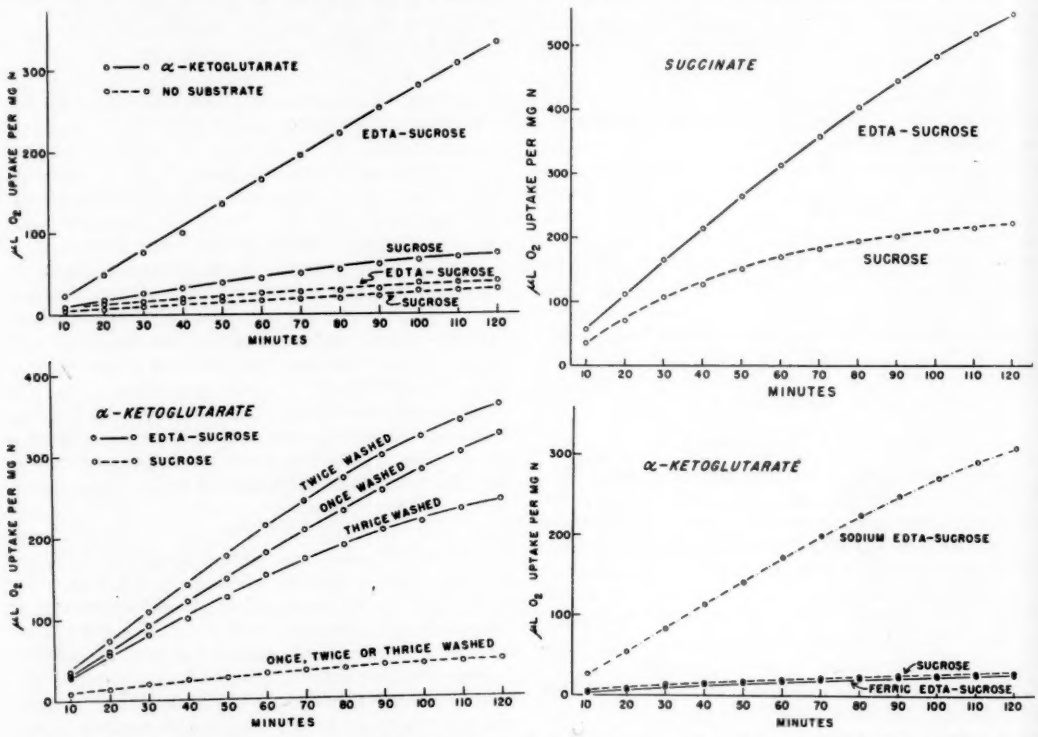

Fig. 1 (*upper left*). Oxidation of α-ketoglutarate by mitochondria from broccoli buds isolated in 0.5 M sucrose medium and in a medium containing 0.01 M EDTA in 0.5 M sucrose at 25° C. Reaction mixture contained 0.001 M ATP, 0.006 M Mg^{++}, 0.02 M glucose, 0.5 M sucrose, 0.02 M α-ketoglutarate, and 0.5 ml mitochondrial suspension containing approximately 1.0 mg nitrogen.

Fig. 2 (*upper right*). Oxidation of succinate by mitochondria from broccoli buds isolated in a 0.5 M sucrose medium and in a medium containing 0.01 M EDTA in 0.5 M sucrose at 25° C. Reaction mixture additions were the same as for figure 1 except for substrate.

Fig. 3 (*lower left*). Comparison of oxidative activity of once-, twice-, and thrice-washed preparations of mitochondria isolated in a 0.5 M sucrose medium and in a medium containing 0.01 M EDTA in 0.5 M sucrose at 25° C. Reaction mixture contained same additions as in figure 1.

Fig. 4 (*lower right*). Comparison of oxidative activity of mitochondria prepared in 0.01 M disodium EDTA in 0.5 M sucrose, 0.01 M ferric EDTA in 0.5 M sucrose, and in 0.5 M sucrose, at 25° C. Reaction mixture contained same additions as in figure 1.

TABLE I

EFFECT OF ADDING DISODIUM EDTA TO REACTION MIXTURE * ON OXIDATIVE ACTIVITY

PREP MEDIUM	NO. OF WASHES	ADDITION OF 0.001 M EDTA TO REACTION MIXTURE	$Q_{O_2}(N)$
0.5 M Sucrose	1	–	36
" "	1	+	37
0.5 M Sucrose	2	–	34
" "	2	+	41
0.5 M Sucrose	3	–	29
" "	3	+	23
0.5 M Sucrose-0.1 M EDTA	2	–	213
" " " "	2	+	232

* Reaction mixture contained same additions as in fig. 1.

stances are salts of EDTA which differ mainly in their capacity to chelate, the activation effect apears to be due to chelation.

EFFECT OF EDTA AFTER ISOLATION OF MITOCHONDRIA IN 0.5 M SUCROSE: Experiments were performed to determine the effectiveness of disodium EDTA in the reaction mixture containing mitochondria prepared with and without EDTA.

It appears from the data shown in table I that 0.001 M disodium EDTA added to the reaction mixture, after isolating the mitochondria in 0.5 M sucrose, has no beneficial effect on oxidation. Addition of 0.001 M disodium EDTA to the reaction mixture after isolating the mitochondria in EDTA had no additional effect. Apparently the chelation which enhances oxidation by the mitochondria must take place during the time the cells are broken. Otherwise the mitochondria are irreversibly inhibited.

DISCUSSION

As a result of this study the generalization can be made that active mitochondria with a stabilized rate of activity could be obtained only by isolating in an EDTA-sucrose medium. It must be pointed out, however, that seasonal variability of the tissue was considerable and during the summer and early fall the stimulatory effect of EDTA was not obtained. In these cases the activity of the isolated mitochondria was extremely low, probably because of factors unrelated to the action of EDTA. The data reported here were obtained from material secured during the winter and spring months.

The simplest explanation of the data herein reported is to assume that EDTA stimulates activity by chelation of inhibitory cations. It is not known specifically what cation or cations are chelated. One can, however, postulate that the deleterious cations, released into the homogenate when the cells are broken, are being sequestered. An example of this type of inhibition is the oxidation of SH groups by heavy metals. In the living cell these cations may be combined in linkages which make their presence innocuous. The physiological shock of cell rupture or the mixing of vacuolar sap with cytoplasmic material may cause hydrolysis of the linkage which binds the cation to its endogenous partner. When freed in the homogenate these cations may become attached to enzymes or cofactors in a manner which inhibits them. However, when the cells are broken in the presence of disodium EDTA, the deleterious ions are chelated and the mitochondria are spared. Once homogenization in the absence of EDTA has occurred, the mitochondria are irreversibly inactivated.

The reports on the use of EDTA with plant tissues are variable. Price and Thimann (10) observed that the addition of EDTA to the extraction medium was of little consequence with mitochondria from etiolated pea stems, but with further purification a definite beneficial effect was shown in succinoxidase activity. Biale and Young (2), using avocado tissue, and Sharpensteen and Conn (13), using white potato, found no beneficial effect of EDTA. Tager (16) reported considerable enhancement of pyruvate oxidation by mitochondria from Avena seedlings prepared in sucrose solutions containing EDTA. From these reports it appears that while EDTA is effective with some plant tissues, it is only partially effective with others and totally ineffective with still others.

Apparently the beneficial effect of EDTA is more universal with animal tissues. Slater and Cleland (14) preserved the activity of heart sarcosomes at temperatures of 15 to 25° C by addition of EDTA to the sarcosomal suspension. The best sarcosomal preparations were obtained by including disodium EDTA in the isolation medium. However, these sarcosomes were very susceptible to inactivation by added calcium ions. Since calcium is known to stimulate ATP-ase (1), Slater and Cleland's concept (15) is that calcium is adsorbed on the sarcosomes during isolation and subsequently causes hydrolysis of the ATP in the membrane. Consequently there is a loss of the high-energy phosphate, which is presumably needed to maintain the stability of the mitochondrial membrane. The presence of EDTA in the isolating medium or in the suspension serves to chelate calcium and thereby prevents the inactivation.

Gross (8) demonstrated that EDTA in concentrations of 0.001 to 0.003 M accelerates ATP-ase activity, but in concentrations above 0.003 M inhibited ATP-ase by chelation of calcium present in a concentration of 0.003 M. The acceleration at lower concentrations of EDTA was attributed to chelation of inhibiting trace metals. The work of Friess (6), Friess et al (7), and Bowen and Kerwin (4) on the effect of EDTA on ATP-ase may have considerable bearing on the concepts of Slater and Cleland (15) and Bonner (3).

Bonner (3) found that succinoxidase preparations from heart muscle, inactivated by various means, could be reactivated by addition of 10^{-4} M EDTA. This was apparently not a chelation effect since inactivation occurred when EDTA was removed from the

preparation by washing or dialyzing against trace-metal-free buffer solutions. Addition of EDTA to this inactivated preparation reactivated it. Inactivation was thus reversible, depending on the presence or absence of EDTA. These data indicate that the activation effect of EDTA might be due to a direct action on the enzyme.

It should be noted that Bonner's succinoxidase system was not a standard mitochondrial preparation. The reversible activation by EDTA in this preparation can be explained by assuming that the inhibiting cation is very firmly fixed to the protein structure and cannot be removed by dialysis. Therefore, EDTA can only be effective when it is in contact with the protein and can mask or shield the enzyme from the inhibiting cation.

Apparently in some tissues the deleterious cations are very firmly bound to the protein complex. In these cases EDTA must be present to block the inhibiting cations. Other tissues, such as broccoli, may have mobile or loosely held inhibiting cations which can be more easily sequestered. In this latter case the effect of EDTA is non-reversible as shown earlier. The avocado (2) may be an example of a third type of tissue in which EDTA has no observable effect. This tissue may be one in which the cation concentration is so low that further chelation is ineffective.

Summary

1. Preparation of broccoli mitochondria in a medium containing 0.01 M disodium EDTA in 0.5 M sucrose stimulates the oxidation of α-ketoglutarate and succinate two- to fourfold and also stabilizes the rate of oxidation during a 2-hour period.

2. a) Removal of EDTA from activated mitochondria by washing did not inactivate them. b) The use of ferric EDTA, a stable salt which has practically no chelating properties, did not cause activation. c) Addition of disodium EDTA to the reaction mixture containing sucrose-prepared mitochondria did not activate them.

3. The data presented indicate that chelation of deleterious cations is involved in the mode of action of disodium EDTA. Once contamination by the deleterious factors has occurred, the inactivation is irreversible with EDTA.

Literature Cited

1. Bailey, K. Myosin and adenosinetriphosphatase. Biochem. Jour. 36: 121. 1942.
2. Biale, J. B. and Young, R. E. Effects of EDTA on oxidation mediated by avocado mitochondria. Abstract of papers, Western Section, Amer. Soc. Plant Physiol. June meeting, 1954.
3. Bonner, W. D. Activation of the succinic dehydrogenase-cytochrome system. Biochem. Jour. 56: 274–285. 1954.
4. Bowen, W. J. and Kerwin, T. D. A study of the effects of EDTA on myosin ATP-ase. Jour. Biol. Chem. 211: 237–247. 1954.
5. Darbey, A. Colorimetric determination of sodium salts of EDTA. Anal. Chem. 24: 373–378. 1952.
6. Friess, E. T. The effects of a chelating agent on myosin ATP-ase. Arch. Biochem. Biophys. 51: 17–23. 1954.
7. Friess, E. T., Morales, M. F., and Bowen, W. J. Some further observations on the interaction of EDTA with myosin-ATP system. Arch. Biochem. Biophys. 53: 311–313. 1954.
8. Gross, M. Effect of EDTA on ATP-ase activity. Science 118: 218–219. 1953.
9. Laties, G. C. The physical environment and oxidative and phosphorylative capacities of higher plant mitochondria. Plant Physiol. 28: 557–575. 1953.
10. Price, C. A. and Thimann, K. V. The estimation of dehydrogenases in plant tissue. Plant Physiol. 29: 113–124. 1954.
11. Schwarzenbach, G. and Ackermann, H. Die Athylendiamin-tetraessigsaure. Helv. Chim. Acta 30: 1798–1804. 1947.
12. Schwarzenbach, G. and Heller, J. Die Eisen (II) und Eisen (III) Komplexe der Athylendiamintetraessigsaure und ihr Redoxgleichgewicht. Helv. Chim. Acta 34: 576–591. 1951.
13. Sharpensteen, H. H. and Conn, E. E. Preparation and properties of potato mitochondria. Abstract, 29th Ann. Meeting Amer. Soc. Plant Physiol. P. 37. Sept. 1954.
14. Slater, E. C. and Cleland, K. W. Stabilization of oxidative phosphorylation in heart muscle sarcosomes. Nature 170: 118–119. 1952.
15. Slater, E. C. and Cleland, K. W. The effect of calcium on the respiratory and phosphorylative activities of heart muscle sarcosomes. Biochem. Jour. 55: 566. 1953.
16. Tager, J. M. The oxidation of pyruvic acid by a particulate fraction from Avena seedlings. Physiol. Plantarum 7: 625–631. 1954.

INFRA-RED ABSORPTION SPECTRA OF CHLOROPHYLLS AND DERIVATIVES[1,2]

A. S. HOLT[3] AND E. E. JACOBS

DEPARTMENT OF BOTANY, UNIVERSITY OF ILLINOIS, URBANA, ILLINOIS

We have measured the infra-red absorption spectra of chlorophyll and some of its derivatives in order to obtain information useful for eventual understanding of reversible transformations of this pigment. Such transformations are (a) Molisch phase test, (b) bleaching by $FeCl_3$, and (c) photochemical reduction by ascorbic acid—"Krasnovsky reaction" (10, 13, 16). Only in the case of the phase test is there evidence of the specific group involved (the cyclopentanone ring). Whether this ring is involved in the other reactions is not known. However, if the C=O bands in the spectrum of the parent substance are correctly assigned, any change in this ring should be apparent from the infra-red spectrum of the intermediate. To obtain unequivocal assignments of the C=O stretching bands we prepared and studied appropriate derivatives. In addition we studied the influence of solvent on the spectrum and found strong effects in the C=O region. These solvent effects were not studied by earlier investigators (18, 19).

The complexity of the spectra below 1600 cm^{-1} is so great that we have not attempted to assign the bands. Spectra are presented from 650 to 3800 cm^{-1} in order to make the data available for future comparisons (except in cases of chlorophyll a in ethyl ether and ethyl pheophorbide a-oxime in $CHCl_3$).

MATERIALS AND METHODS

Preparative techniques for most of the pigments studied have been described by us elsewhere (5, 6, 8). Those not described are as follows:

1. Pheophorbide a: Two-tenths gm of pheophytin a were incubated in a mixture of 80 % acetone (v/v, aqueous) and pigment-free meal obtained from leaves of *Ailanthus altissima*. Complete removal of phytol by chlorophyllase after 4 hours was demonstrated by transferring a test sample into ethyl ether and extracting with 0.01 *N* KOH (22). The entire pigment was then transferred into ether, dried, and adsorbed on a powdered sucrose column from 40 % $CHCl_3$ + 60 % petroleum ether mixture. The column was treated first with benzene, and then with 0.05, 1.0 and 2.0 % isopropanol in petroleum ether. The sugar bearing the main part of the pigment was removed and washed with petroleum ether to remove residual chloroform and benzene. The pigment was eluted from the sugar with acetone, transferred into ethyl ether, dried and subjected again to chromatographic purification. The product gave a positive Molisch phase test and had an absorption spectrum in the visible region identical with the known spectrum of ethyl pheophorbide a (5).

2. Pyropheophorbide a: This compound was prepared according to Fischer and Siebel (3). A solution of pheophorbide a in pyridine containing Na_2CO_3 was refluxed for 3 hours. The pigment was transferred into ethyl ether and extracted three times with 10 % HCl to remove phylloerythrin. A fraction was then extracted with 14 % HCl, transferred back into ethyl ether, dried in vacuo and adsorbed on a powdered sucrose column from 40 % $CHCl_3$ + 60 % petroleum ether. The column was treated first with benzene and then with 0.5 % isopropanol + 30 % chloroform in petroleum ether. The pigment-bearing zone was removed and the pigment was eluted and re-purified by chromatography.

3. Mono-2,4-dinitrophenylhydrazine derivatives of the following substances were prepared by a modification of the procedure of Iddles et al (7):

(a) Pheophorbide a: Two ml of 99.5 % ethanol saturated with 2,4-dinitrophenylhydrazine were acidified with 0.1 ml of 6 *N* HCl, and added to 20 mg of pheophorbide a dissolved in 2 ml of ethanol; the mixture was boiled for 2 minutes. A precipitate, which formed overnight at room temperature, was collected by centrifugation; it was washed twice with 75 % ethanol (aqueous) and dried in vacuo.

(b) Ethyl pheophorbide b: Twenty mg of ethyl pheophorbide b in 6 ml of 99.5 % ethanol were added to an equal volume of a saturated ethanol solution of 2,4-dinitrophenylhydrazine. The mixture was acidified with 0.04 ml of 6 *N* HCl and boiled for 2 minutes. After storage at room temperature for 3 hours, the precipitate was collected, washed twice with 75 % ethanol (aqueous) and dried. According to analysis this procedure gave only the mono-derivative:

	Percent		
	C	H	N
Calculated	62.8	5.31	13.7
Observed	63.5	4.96	13.8

(c) Pyropheophorbide a: This compound was prepared in the same manner as the derivative of pheophorbide a.

4. Ethyl pheophorbide a-oxime: This was prepared according to the method of Fischer et al (2). Forty-two mg of ethyl pheophorbide a were dissolved in 2.5 ml of pyridine containing 0.33 gm of $NH_2OH \cdot HCl$, and allowed to stand 8 days in darkness at room temperature. The product was transferred into ether, and the solution was extracted first with 10 % HCl, and then with 14 % HCl. The pigment extracted with 14 % HCl was transferred back into ether and

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[3] Present address: Division of Applied Biology, National Research Council, Ottawa 2, Ontario, Canada.

dried. Its spectrum was measured without further purification.

The infra-red spectra were measured on a Perkin-Elmer Model 21 Double Beam Recording Spectrophotometer. Matched, sealed absorption cells with rock salt windows were used; the solvents were reagent grade. In the case of the phyllins no noticeable decomposition occurred during the measurement. This was shown by two methods: (a) measuring the infra-red absorption spectrum of a sample twice with an interval of 1 hour between measurements; and (b) by comparing the "purity ratios" (23) in the visible absorption spectra, before and after the measurement. The accuracy of the concentrations (w/v) indicated in the legends of the absorption curves was ± 5 %. Frequencies assigned to the bands in table I are correct to ± 2 cm^{-1} from 650 to 2000 cm^{-1} and to ± 10 cm^{-1} from 2000 to 3800 cm^{-1}.

Structural formulae of the compounds studied are those given by Fischer and Stern (4) and are indicated in figure 1.

Results and Interpretations

Table I presents a summary of the bands assigned.

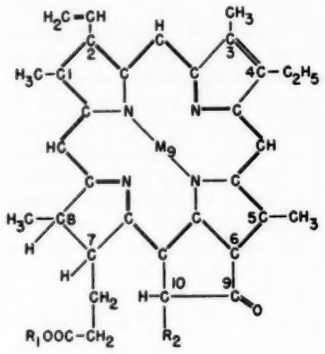
Spectra of Pheophytin and Its Derivatives: Pheophytin a and ethyl pheophorbide a contain three C=O groups—the ketone at C_9 in the cyclopentanone ring, and the propionate and carbmethoxy groups at C_7 and C_{10}, respectively. Two C=O stretching bands can be anticipated.

Ethyl pheophorbide a (in $CHCl_3$—fig 2), pheophorbide a (in $CHCl_3$—fig 3), and pheophytin a (in pyridine and in CCl_4—fig 4) show in fact two C=O bands. A third band in the double bond region, at 1618 to 1620 cm^{-1}, is due to a C=C vibration.

In figure 3, we see that the oxime of ethyl pheophorbide a has, as expected, only one C=O band, and that its maximum lies at 1737 cm^{-1}. The absence of the 1697 cm^{-1} band which appears in the spectrum of ethyl pheophorbide a confirms that this band is attributable to the ketone C=O. the C_7 and C_{10} ester C=O groups must account for the 1737 cm^{-1} band.

The spectrum of pheophorbide a in the C=O region differed from that of ethyl pheophorbide a. The ketone C=O band appeared broadened and shifted to 1702 cm^{-1}. This suggested that the C=O in the carboxyl group of the propionic acid absorbs on the high frequency side of the ketone band. The presence of the carboxyl group is confirmed by the broad OH stretching band between 2400 and 2800 cm^{-1} (1). In the spectrum of the phenylhydrazine derivative of pheophorbide a the most intense C=O band appears at 1728 cm^{-1}; it must be assigned to the one remaining ester group—the C_{10} carbmethoxy group. The shoulder at 1705 cm^{-1} must be due to the band of the carboxyl C=O of the C_7 propionic acid. This assignment agrees well with the observations of Jones et al (9) in studies of steroid acids.

In the spectrum of pyropheophorbide a (which lacks the C_{10} carbmethoxy group) a shoulder at 1704 cm^{-1}, superimposed upon the more intense ketone band at 1685 cm^{-1}, must again be attributed to the propionic acid carboxyl C=O. In the phenylhydrazine derivative of the same compound, only the C=O of propionic acid remains. Unfortunately, this deriva-



COMPOUND	Mg	R_1	R_2
CHLOROPHYLL a	+	$C_{20}H_{39}$	$-C(=O)-OCH_3$
PHEOPHYTIN a	−	"	"
ETHYL CHLOROPHYLLIDE a	+	C_2H_5	"
ETHYL PHEOPHORBIDE a	−	"	"
PHEOPHORBIDE a	−	H	"
PYROPHEOPHORBIDE a	−	H	H
b COMPOUNDS SAME AS ABOVE EXCEPT C_3 METHYL GROUP = $-C(=O)-H$			
C_2 VINYL = $-C(=O)-CH_3$, $\Delta^{3,4}$ SATURATED			
BACTERIOCHLOROPHYLL	+	$C_{20}H_{39}$	$-C(=O)-O-CH_3$
METHYL BACTERIOCHLOROPHYLLIDE	+	CH_3	"

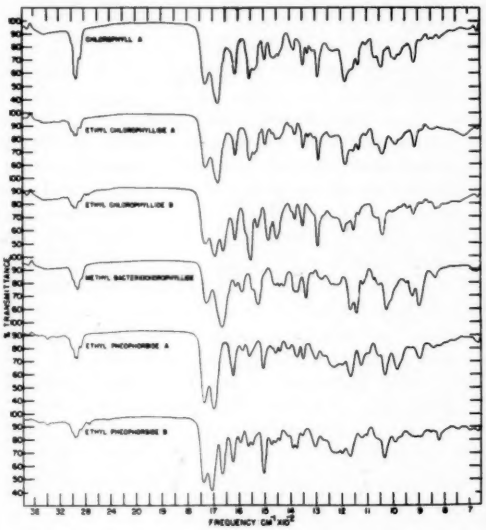


Fig. 1 (*above*). Structure of chlorophylls and derivatives.

Fig. 2 (*below*). Infra-red absorption spectra of chlorophyll and derivatives. Solvent—$CHCl_3$. Concentration—2 %. Cell depth—0.2 mm.

tive was not sufficiently soluble in $CHCl_3$, and its spectrum had to be measured from Nujol (liquid petrolatum) mull. The carboxyl C=O band was found broadened and shifted to 1725 cm^{-1}, probably due to the solid state (1).

The assignment of the 1610 cm^{-1} band to semi-isolated or vinyl C=C (19), is not confirmed by the spectra of the phenylhydrazine derivatives. The intensified absorption at 1613 to 1618 cm^{-1} with these compounds is due to conjugated C=C bonds in the phenyl group. This supports the assignment of this band in the spectra of the phyllins and phytins to conjugated C=C, since the same band is seen also in the spectrum of bacteriochlorophyll (fig 6).

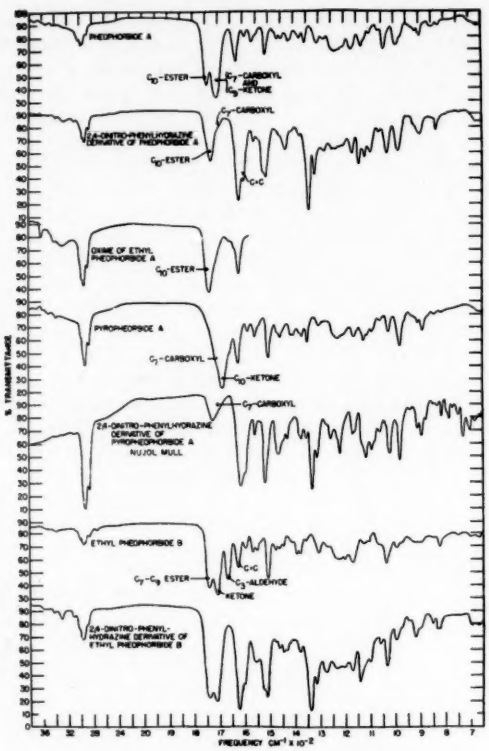
The spectra of pheophytin a in the two solvents CCl_4 and pyridine, are quite similar in the double bond stretching region, but are very different in the higher frequencies. In pyridine (fig 4) we note a broad band between 3700 and 3100 cm^{-1} with a more narrow band superimposed on it at 3382 cm^{-1}. We attribute this double band to vibrations of two different groups—the N—H groups of pyrrole nuclei accounting for the sharp maximum at 3382 cm^{-1}, and a H-bonded or otherwise associated OH group accounting for the broad band. An associated OH group can result from enolization between C_9 and C_{10} and strong bonding of the enolic OH at C_9 with N atom of pyridine (compare with the interpretation of the chlorophyll a spectrum in CCl_4 or CS_2 below). In the spectrum of pheophytin a in CCl_4 (fig 4) the weak band at 3392 cm^{-1} can be attributed to N—H stretching in agreement with the assignment of Weigl and Livingston (19). Phytol causes the strong C—H stretching band at 2920 cm^{-1}.

The band of the C_3 aldehyde group of ethyl pheophorbide b absorbs at 1663 cm^{-1} as proved by its disappearance from the spectrum of the phenylhydrazine derivative and its absence from the spectra of pheophorbide a and ethyl pheophorbide a (fig 2, 3). The characteristic weak C—H stretching band at 2720

TABLE I

INFRA-RED ABSORPTION BANDS OF CHLOROPHYLLS AND DERIVATIVES. FREQUENCIES CORRECTED (CM^{-1})

COMPOUND	SOLVENT	NH	OH	C—H PHYTOL	C—H ALDEHYDE	C=O ESTER	C=O KETONE	C=O CARBOXYL C_7	C=O ALDEHYDE	C=O CHELATE	C=C
Ethyl chlorophyllide a	$CHCl_3$	....	....	....	....	1733	1678	....	....	....	1610
Chlorophyll a	Crystals in Nujol	3605?	3440+ 3252	2920	....	1727+ 1740	1694	....	....	1640	1604
Chlorophyll a	CCl_4, CS_2	....	3350 (weak)	2916	....	1735	1691	....	....	1652	1610
Chlorophyll a	Ethyl ether	....	....	....	....	1738	1698	....	....	....	1608
Chlorophyll a	Pyridine	....	3400 (strong)	....	....	1740	1683	....	....	....	....
Ethyl chlorophyllide b	$CHCl_3$	....	....	....	2720	1730	1689	....	1657	....	1610
Chlorophyll b	$CHCl_3$	....	....	2920	2720	1728	1688	....	1655	....	1610
Chlorophyll b	Crystals in Nujol	....	3360	2924	2710	1738	1697	....	1655	....	1607
Chlorophyll b	CCl_4, CS_2	....	3350	2924	2710	1738	1701	....	1664	....	1609
Pheophytin a	CCl_4	3392	....	2930	....	1740	1705	....	....	....	1618
Pheophytin a	Pyridine	3382	3100-3700	2920	....	1736	1700	....	....	....	1617
Ethyl pheophorbide a	CS_2	3375	....	....	....	1743	1706	....	....	....	1618
Ethyl pheophorbide a	$CHCl_3$	3380	....	....	....	1736	1697	....	....	....	1620
Pheophorbide a	$CHCl_3$	....	2400-2800	....	....	C_{10}: 1741	1702	1702	....	....	1622
Pheophorbide a-2,4-dinitrophenyl-hydrazone	$CHCl_3$	....	....	....	....	C_{10}: 1728	....	1705	....	....	1613
Pyropheophorbide a	$CHCl_3$	....	....	....	....	....	1685	1704	....	....	1620
Pyropheophorbide a-2,4-dinitrophenyl-hydrazone	Nujol mull	....	....	....	....	....	....	1725	....	....	1610
Pheophytin b	CCl_4	3410	....	2930	2720	1740	1709	....	1665	....	1618
Ethyl pheophorbide b	$CHCl_3$	3380	....	....	2720	1737	1704	....	1663	....	1620
Ethyl pheophorbide b-2,4-dinitrophenyl-hydrazone	$CHCl_3$	....	....	....	....	1740	1710	....	....	....	1618
						ester	*ketone*		*acetyl*		
Bacteriochlorophyll	CCl_4	....	3350	2916	....	1735	1683		1655		1610
Methyl bacteriochlorophyllide	$CHCl_3$	....	....	....	....	1727	1662		1662		1610



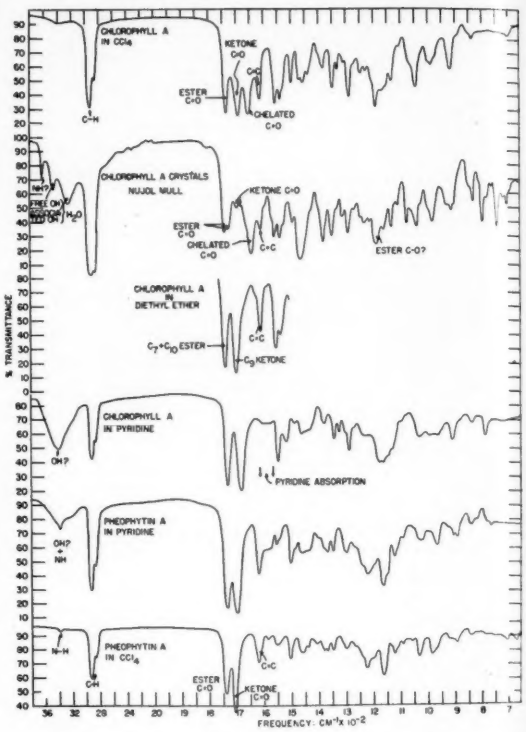


FIG. 3 (*left*). Infra-red absorption spectra of chlorophyll derivatives. Solvent—$CHCl_3$. Cell depth—0.1 mm. Concentrations (starting with uppermost curve)—4 %, 4 %, 3.5 %, 4 %, Nujol mull, 2 %, 6 %.

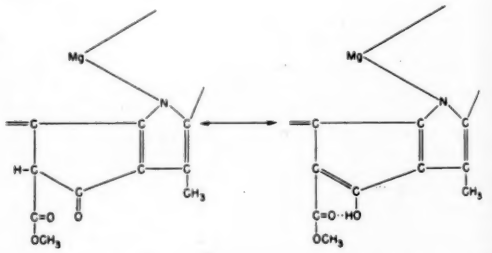
FIG. 4 (*right*). Infra-red absorption spectra of chlorophyll a and pheophytin a in different solvents. Cell depth—0.1 mm. Concentrations—Chlorophyll a (CCl_4—6 %; ethyl ether—6 %; pyridine—3 %), pheophytin a (CCl_4—6 %; pyridine—6 %).

cm^{-1}, seen in figure 5 (15) is further confirmation of the presence of an aldehyde group.

SPECTRA OF PHYLLINS AND DERIVATIVES: Except at 2920 cm^{-1} where C—H groups of phytol absorb strongly, the spectra of chlorophyll a and its ethyl derivative are quite similar (fig 2). As with pheophytin a in $CHCl_3$ the ester C=O groups absorb at 1735 to 1740 cm^{-1}. The ketone band is, however, shifted to 1678 cm^{-1} (a shift of 19 cm^{-1} to lower frequencies) and the C=C band to 1610 cm^{-1} (a shift of 7 to 8 cm^{-1} to lower frequencies).

The aldehyde C=O of ethyl chlorophyllide b absorbs at 1657 cm^{-1}; other double bond bands lie at 1730, 1689 and 1610 cm^{-1} (see fig 5 for spectrum of chlorophyll b in $CHCl_3$).

In figure 4 are the spectra of chlorophyll a in CCl_4, pyridine and ethyl ether; the spectrum of crystalline chlorophyll a dispersed in Nujol is also included. We note that in CCl_4 solution four bands occur between 1735 and 1610 cm^{-1}, in contrast to only three in $CHCl_3$ or ether solutions (only two are visible in pyridine solution, since the band at ca 1610 cm^{-1} is blocked by the intense absorption of pyridine itself). Pheophytin a in CCl_4 (fig 4) shows only three bands. We believe this extra band, which appears only when chlorophyll a (or ethyl chlorophyllide a), but not pheophytin a, is dissolved in non-polar solvents (CS_2, CCl_4), results from enolization and chelation, as follows:



Precedent for this interpretation is found in studies of Leonard et al (11) dealing with chelation in β keto esters of cyclopentanone, e.g., diethyl cyclopentanone-2-5-dicarboxylate. In the spectrum of this compound an additional band of medium intensity was observed at 1671 cm^{-1} and assigned to chelated C=O. The absence of an enolic OH band in the spectra of keto

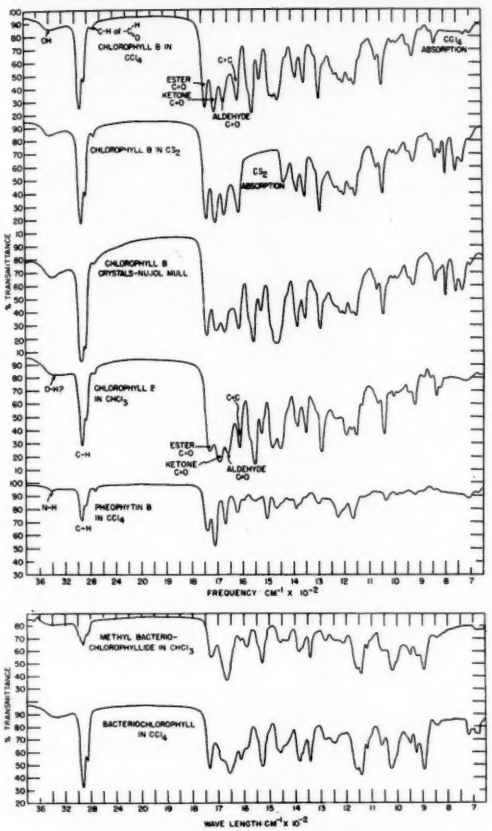


FIG. 5 (*above*). Infra-red absorption spectra of chlorophyll b and pheophytin b in different solvents. Cell depth—0.1 mm. Concentrations: Chlorophyll b (CCl_4—6 %; CS_2—7.4 %; $CHCl_3$—6 %), pheophytin b—3 %.

FIG. 6 (*below*). Infra-red absorption spectra of methyl bacteriochlorophyllide in $CHCl_3$ (2 %) and bacteriochlorophyll in CCl_4 (5 %). Cell depth—0.1 mm.

esters of cyclohexanone also was noted by these workers, and interpreted as a confirmation that the enol existed chiefly in the chelated form. The weak broad OH band at ca 3350 cm^{-1} seen in the spectrum of chlorophyll a may indicate the presence of free enolic OH, but may also be due to bound or associated H_2O molecules. This possibility is suggested by our earlier conclusion that water is necessary for crystallization of chlorophyll a, b and bacteriochlorophyll (8), and also by the presence of bands of free and associated OH in the spectrum of crystalline chlorophyll a dispersed in Nujol. From the much greater intensity of absorption by chelated C=O relative to that by the ester or ketone C=O groups, we infer that in the crystalline state, chlorophyll a exists almost entirely in the chelated form. A sharp band at 3605 cm^{-1}, which is absent in the spectra of solutions, is noticeable also in the spectrum of the crystals. We have tentatively assigned it to N—H groups in the pyrrole nuclei, but cannot explain the shift from 3392 cm^{-1} where the N—H band is located in solutions of pheophytin a; nor can we account for its increased intensity.

In pyridine, chlorophyll a shows evidence of a very strong associated OH band, and no evidence of a chelated C=O band. It appears that enolization has occurred, but that chelation of the β keto ester has been hindered by strong bonding between the enolic OH group at C_9 and the nitrogen atom in pyridine.

Figure 5 shows the spectra of crystals of chlorophyll b in Nujol, and of chlorophyll b dissolved in CCl_4, CS_2, and $CHCl_3$. In all spectra we note four bands in the region 1735 to 1600 cm^{-1}, which, from the evidence given above are characteristic respectively of ester, ketone, and aldehyde C=O groups and of C=C bonds. We note also that the spectrum of crystals in Nujol is very similar to the solution spectra. This is in contrast to the case of chlorophyll a for which the spectrum of crystals is quite different, in the C=O region, from the spectra in polar solvents. We found but one sample of chlorophyll b with an additional band that may be ascribed to chelated C=O; this band was located at 1645 cm^{-1}. On further chromatographic purification and recrystallization (shown by X-ray diffraction) this band no longer appeared. Twenty subsequent spectra gave no evidence of this band. The consistency of these results suggests that chlorophyll b does not enolize or chelate, in CCl_4, CS_2 or when crystallized. Because an OH bending vibration occurs at 1642 cm^{-1} we first surmised that the extra band seen with the earlier sample was due to water (17). However, while the addition of water to solutions of chlorophyll b in CCl_4 increased the absorption at this frequency, it did not produce the originally observed sharp band.

The spectrum of bacteriochlorophyll in CCl_4 is shown in figure 6 with that of methyl bacteriochlorophyllide in $CHCl_3$. Here again we see considerable difference between spectra in the two solvents. Two bands in the double bond region can be assigned with assurance—the ester C=O band at 1735 cm^{-1} (CCl_4), 1727 cm^{-1} ($CHCl_3$) and the C=C band at 1610 cm^{-1}. In $CHCl_3$ the intense band at 1662 cm^{-1} must be assigned to the ketone groups at C_9 and C_2. In CCl_4, we note a separation of this band into two bands, the more intense one lying at 1655 cm^{-1} and the other broadened one, lying at 1683 cm^{-1}. Unfortunately, we did not have sufficient pigment to prepare derivatives which would permit us to distinguish between the cyclopentanone and the acetyl C=O groups. Tentatively, we assign the band at 1685 cm^{-1} to the cyclopentanone C=O, and the more intense band at 1657 cm^{-1} to the conjugated acetyl C=O. However, the reverse assignments may also be possible, in which case bacteriochlorophyll in CCl_4 would have the chelated-enol form. Further work must be done to clarify the assignments of the C=O bands of this compound.

Discussion

From the spectra described above we see that the β keto ester of the cyclopentanone ring of chlorophyll a is enolized and chelated, both in the crystalline state and in CCl_4 and CS_2. This is not the case when the same pigment is dissolved in other solvents, e.g., $CHCl_3$ or ether. In pyridine it appears that the pigment is enolized but not chelated. The spectrum of pheophytin a in CCl_4 shows that the removal of magnesium from chlorophyll a prevents enol formation, except in basic solvents such as pyridine. This result is interesting and invites speculation. Since magnesium is less electronegative than the hydrogen by which it is replaced in pheophytin (14), it follows that the C_9 keto oxygen will be more negative when magnesium is present, and enolization will be facilitated.

The reason why chlorophyll b does not enolize and chelate in CCl_4 may be because the electronegative C_3 aldehyde group can reduce the ability of magnesium to induce a negative charge on the C_9 keto oxygen.

It is also known that in anhydrous alcohol chlorophyll a is allomerized more rapidly than chlorophyll b, and that pheophytins a and b are not allomerized (4). Such results would be expected if formation of the enol is prerequisite to production of the unstable intermediate which reacts with oxygen. Weller and Livingston (20, 21) hypothesize that this intermediate is produced as the result of traces of base present in the alcohol and that it is actually the phase-test intermediate.

The fact that chlorophyll b does not chelate in CCl_4 may also be due to hydrogen bonding between traces of polar substances, e.g., H_2O and the C=O groups of the β keto ester. Livingston et al (12) have suggested that such an addition is responsible for the activation of chlorophyll fluorescence. The keto form is presumed to be fluorescent, and the chelated-enol form to be non-fluorescent. This hypothesis could now be tested by measurement of the infra-red spectrum of chlorophyll b dissolved in the specially purified benzene used by these workers. A chelated C=O band should appear if the non-fluorescent form is a chelated enol.

Summary

1. C=O, C=C, C—H and N—H groups in chlorophylls and their derivatives have characteristic infra-red absorption bands. In the double bond stretching region the absorption bands of the following groups have been assigned: C_7 and C_{10} ester C=O; C_9 ketone C=O; C_7 carboxyl C=O of the free acid; C_3 aldehyde C=O of the b series; conjugated C=C.

2. When chlorophyll a is dissolved in the polar solvents $CHCl_3$ and ethyl ether, the β keto ester of the cyclopentanone ring exists in the keto form. In pyridine, the enol form is present as shown by a strong associated OH stretching band. In the non-polar solvents CCl_4 and CS_2, and when the pigment is crystalline, the enol form is chelated, as shown by the appearance of a chelate C=O stretching band.

3. Pheophytin a shows neither a chelate C=O band nor an enolic OH band when dissolved in CCl_4 or CS_2. An enolic OH band appears in pyridine solution.

4. Chlorophyll b does not enolize and chelate when dissolved in $CHCl_3$, CCl_4 or CS_2, nor when it crystallizes.

5. It is suggested that the C_9 keto oxygen is more negatively charged (thus facilitating enolization) when magnesium is present than when it is absent as in pheophytin, and that it is more negatively charged in chlorophyll a than it is in chlorophyll b.

The authors are indebted to Dr. E. I. Rabinowitch, Dr. R. Emerson, Dr. N. J. Leonard, Dr. H. S. Gutowsky, and Mr. J. Brader (who measured the infra-red spectra) for many helpful suggestions during this work.

Literature Cited

1. Bellamy, L. J. The Infra-red Spectra of Complex Molecules. Pp. 1–323. Methuen, London. 1954.
2. Fischer, H., Riedmair, J., and Hasenkamp, J. Über Oxo-porphyrine: Ein Beitrag zur Kenntnis der Feinstruktur von Chlorophyll a. Annalen Chemie, Justus Liebigs 508: 224–249. 1933.
3. Fischer, H. and Siebel, H. Überführung von Chlorin e-trimethylester in Desoxy-pyro-phäophorbid. Annalen Chemie, Justus Liebigs 474: 73–86. 1932.
4. Fischer, H. and Stern, A. Chemie des Pyrrols. vol. II, 2. Pp. 1–478. Akadem. Verlagsgesellschaft, Leipzig. 1940.
5. Holt, A. S. and Jacobs, E. E. Spectroscopy of plant pigments. I. Ethyl chlorophyllides a and b and their pheophorbides. Amer. Jour. Bot. 41: 710–717. 1954.
6. Holt, A. S. and Jacobs, E. E. Spectroscopy of plant pigments. II. Methyl bacteriochlorophyllide and bacteriochlorophyll. Amer. Jour. Bot. 41: 718–722. 1954.
7. Iddles, H. A., Low, A. W., Rosen, B. D., and Hart, R. T. Determination of carbonyl compounds by means of 2,4-dinitrophenylhydrazine—water insoluble compounds. Ind. Eng. Chem., Anal. Ed. 11: 102–103. 1939.
8. Jacobs, E. E., Vatter, A. E., and Holt, A. S. Crystalline chlorophyll and bacteriochlorophyll. Arch. Biochem. Biophys. 53: 228–238. 1954.
9. Jones, R. N., Humphries, P., and Dobriner, K. Studies in steroid metabolism. IX. Further observations on the infrared absorption spectra of keto-steroids and steroid esters. Jour. Amer. Chem. Soc. 72: 956–961. 1950.
10. Krasnovsky, A. A. Reversible photochemical reduction of chlorophyll with ascorbic acid. Doklady Akad. Nauk SSSR. 60: 421–424. 1948.
11. Leonard, N. J., Gutowsky, H. S., Middleton, W. J., and Peterson, E. M. The infrared absorption spectra of cyclic β-ketoesters. Jour. Amer. Chem. Soc. 74: 4070–4073. 1952.
12. Livingston, R., Watson, W. F., and McArdle, J. Activation of the fluorescence of chlorophyll solutions. Jour. Amer. Chem. Soc. 71: 1542–1550. 1949.
13. Molisch, H. Eine neue mikrochemische Reaktion auf Chlorophyll. Ber. deut. bot. Ges. 14: 16–18. 1896.

14. Pauling, L. The Nature of the Chemical Bond. Pp. 1–450. Cornell Univ. Press, Ithaca, New York. 1940.

15. Pinchas, S. Infrared absorption of the aldehydic C—H group. Anal. Chem. 27: 2–6. 1955.

16. Rabinowitch, E. and Weiss, J. Proc. Roy. Soc. (London) A 162: 251–267. 1937.

17. Randall, H. M., Fowler, R. G., Fuson, N., and Dangl, J. R. Infrared Determination of Organic Structures. Pp. 1–239. D. Van Nostrand, Inc, London. 1949.

18. Stair, R. and Coblentz, W. W. Infrared absorption spectrum of some plant pigments. Jour. Research Natl. Bur. Standards 11: 703–711. 1933.

19. Weigl, J. W. and Livingston, R. Infrared spectra of chlorophyll and related compounds. Jour. Amer. Chem. Soc. 75: 2173–2176. 1953.

20. Weller, A. The visible absorption spectra of the phase test intermediates of chlorophyll a and b. Jour. Amer. Chem. Soc. 76: 5819–5821. 1954.

21. Weller, A. and Livingston, R. The reaction of chlorophyll with amines. Jour. Amer. Chem. Soc. 76: 1575–1578. 1954.

22. Willstätter, R. and Stoll, A. Untersuchungen über Chlorophyll. Pp. 1–424. J. Springer, Berlin. 1913.

23. Zscheile, F. P. and Comar, C. L. Influence of preparative procedure on the purity of chlorophyll components as shown by absorption spectra. Bot. Gaz. 102: 463–481. 1941.

BRIEF PAPERS

HYDROSTATIC PRESSURE IN COCONUTS[1,2]

P. F. SCHOLANDER[3]
WOODS HOLE OCEANOGRAPHIC INSTITUTION
AND
LERNER MARINE LABORATORY, BIMINI, BAHAMAS

David Fairchild in "Garden Islands of the Great East" (3) gives a vivid description of the natives of Java and the Philippines climbing wine palms 60 feet high and harvesting every day a bamboo container full of the sugary sap which drips from the bruised and cut stems of the inflorescences. The coconut palm also yields enough sap from the cut flower stems to support an important palm sugar industry (2). As the pressure in the transpiration stream of the palm stem is very likely low, and possibly under cohesive tension, some local process must develop in the cut flower stem which steps up this pressure to make it possible for sap to drip out 20 meters above the ground.

It is described as quite an art to get a successful flow of sap from the coconut palm, but a large supply of free liquid is readily available inside the coconuts, and one might wonder what the pressure of this liquid would be. During a visit to the Lerner Marine Laboratory in Bimini, Bahamas, an opportunity arose to find out. A green, unripe coconut was taken into the laboratory, and a syringe needle pushed into the cavity. When the needle was pulled out a fine jet of sap shot out of the puncture hole with such a force that it hit the ceiling. This occasioned some studies of the pressure relations of the coconut milk in respect to the size of the nut, its height above ground, and the osmotic potential of the milk. By comparing these values one may venture to make certain guesses as to the nature of the mechanism responsible for the pressure build-up in the nuts.

It was established that young nuts in the same inflorescence, i.e, of the same age and of selected size, had similar pressures whether measured in situ or freshly detached. No liquid leaked from the cut petiole. The pressure was measured in the laboratory with a tuberculin syringe full of water and containing

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[2] Contribution No. 822 from the Woods Hole Oceanographic Institution.
[3] Present address: Institute of Zoophysiology, University of Oslo, Blindern, Norway.

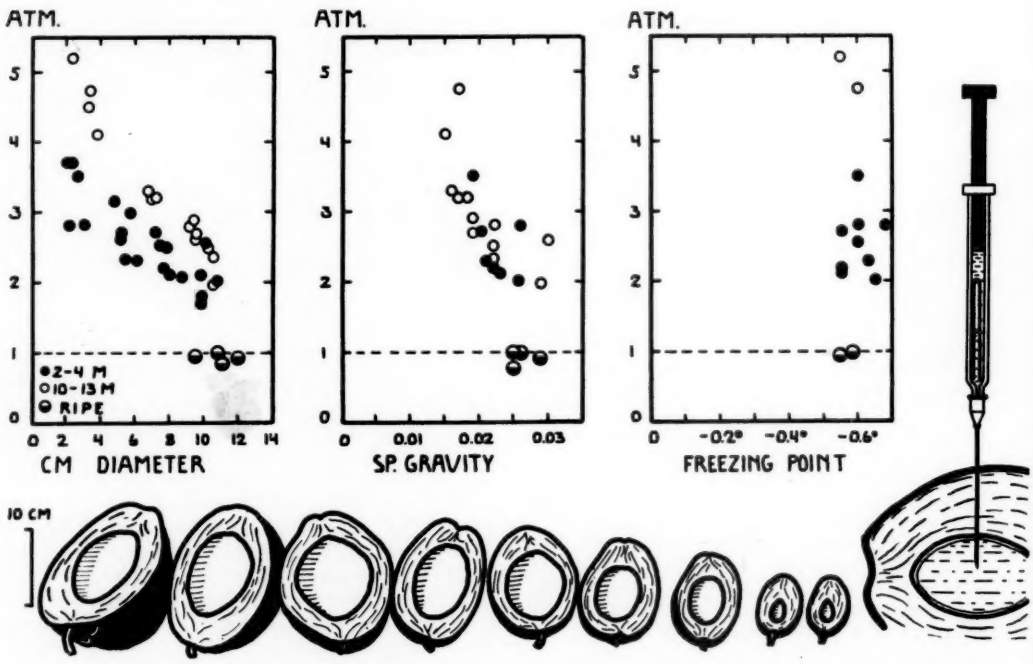

FIG. 1. *Top.* Liquid pressure in coconut cavity versus (a) largest diameter of cavity, (b) specific gravity of milk, (c) freezing point of milk. *Bottom.* Size range of nuts used, after photograph. *Right.* Method for pressure measurements. Capillary manometer inside syringe barrel.

a small air-filled capillary manometer open at one end (fig 1). The length of the air column in the capillary was measured on the syringe scale (V_1), and the position of the plunger was noted. The needle was pushed into the cavity of the nut and unplugged by forcing the plunger in slightly. The plunger was immediately returned to its original position and held there while the gas volume in the manometer tube was measured (V_2). The hydrostatic pressure was calculated as V_1/V_2 in atmospheres. The pressure dropped slowly, especially in the smallest nuts, due to a slight leak along the needle. The figures are hence minimum values, but undoubtedly quite near to the true values. In ripe, hard-shelled nuts it was necessary to drill through the husk and hard outer shell before introducing the needle.

Freezing point determinations were performed by observing the melting and freezing of a few microscopic ice crystals present in a large excess of sap, according to a modification of the method described by Pounder and Masson (4).

Specific gravity of the coconut sap was determined by weighing in a pycnometer.

In young green nuts the hydrostatic pressure increases with decreasing size (fig 1). The nuts which were taken from two tall palms 10 and 13 meters above ground had higher pressures in all sizes than were found in nuts taken from lower palms at 2 to 4 meters. The values ranged from 2 atmospheres in the biggest green nuts to over 5 atmospheres in the smallest. Only ripe nuts contained a gas phase, usually somewhat below atmospheric pressure, suggesting the possibility that part of the milk had been absorbed and that gas had diffused in to take its place.

The freezing point of the coconut milk was between $-0.55°$ and $-0.68°$ C, with an average osmotic potential of 7 atmospheres. This was not correlated with milk pressure, specific gravity, height above ground, or size (age) of the nuts (fig 1).

The specific gravity of the coconut milk varied between 1.015 and 1.030, with the lowest values in the sap which was under the highest pressure (fig 1). This correlation undoubtedly is secondary to the fact that the sap gets slightly more concentrated as the nuts grow older (1). Compared to the very watery coconut milk, the palm sugar sap dripping from the cut flower stems has been found to be some three times more concentrated, with a specific gravity averaging 1.07 (2).

There is an inverse relation between the size of the nut cavity and the internal pressure. This is not very surprising on the basis of structural stress considerations, for the maximum pressure which a hollow sphere can stand for a given wall strength is inversely proportional to the diameter.

Coconuts growing on the same tree are equally high above the ground and ultimately derive water from the same source, namely, the transpiration stream in the palm trunk, which for all the nuts may be assumed to present the same osmotic potential. The coconuts were all found to have essentially the same osmotic potential in the milk. With all these factors constant, if the nuts were simple osmometers, they should have the same hydrostatic pressure. But this was found to be related to the nut size and varied from 5 atmospheres in the small nuts to 2 atmospheres in the large ones. Some of these isosmotic nuts did therefore maintain an osmotic unbalance of at least 3 atmospheres, i.e, the hydrostatic pressure in the nuts is not produced by a simple process of osmosis. The fact that the hydrostatic pressure in isosmotic nuts was higher in tall palms than in low, points in the same direction. One may furthermore observe that it seems dubious that an osmotic "pull" of 7 atmospheres by the coconut milk would be enough to produce a pressure of 5 atmospheres in the nuts, against: a hydrostatic pull of 13 meters plus whatever cohesive and osmotic "pull" there may be in the sap of the palm stem.

One may therefore conclude that there must be some active "secretory" process capable of regulating the pressure in the coconut against an osmotic gradient of several atmospheres. As detached nuts do not bleed through the stalk, it seems likely that the pressure regulation is located within the nut.

Literature Cited

1. Chandrasena, J. P. C. The chemistry of the products of *Cocos nucifera*. II. Biochem. Jour. **27**: 3–4. 1933.
2. Copeland, E. B. The Coconut, 3rd ed, rev. Pp. 1–233. Macmillan and Co., Ltd., London 1931.
3. Fairchild, D. Garden Islands of the Great East. Pp. 1–239. Charles Scribner's Sons, New York 1943.
4. Pounder, F. E. and Masson, I. Thermal analysis and its application to the dinitrobenzenes. Jour. Chem. Soc. 1934: 1357–1360. 1934.

EFFECT OF AQUEOUS PERCHLORIC ACID ON THE XYLAN CONTENT OF HOLOCELLULOSE FROM CORNSTALKS[1,2]

EMMETT BENNETT
MASSACHUSETTS AGRICULTURAL EXPERIMENT STATION,
AMHERST, MASSACHUSETTS

Hemicelluloses are not easily removed completely from plant material by any solvent. Preliminary tests in this laboratory indicated that isolated hemicelluloses were reasonably soluble in aqueous perchloric acid. Because of this observation, the difficulties in extraction, and the fact that this solvent is satisfactory for starch (1, 2) it was of interest to determine the possibilities of perchloric as a solvent for hemicelluloses.

Holocellulose prepared from cornstalks (3) was used as source material. Five molar and 2.5 M perchloric acid were used for extractions. The content of furfural calculated as xylan and the loss in total weight of the samples were used to indicate the progress of the reaction which was carried out as follows: a 0.35 gm charge of holocellulose in triplicate was weighed in tubes 28 × 160 mm and placed in a constant temperature water bath operating at 25° C. The tubes were equipped with a motor-driven stirrer which operated during the entire solubility test. Fifteen ml of 5 or 2.5 M perchloric were added from a pipette while the sample was stirred. Solubility tests were conducted for 15, 30, 45, 60, 90 and 120 minutes. Control samples were obtained by extracting the sample with water for the same period of time. At the end of each trial the sample was filtered and washed with ethanol, ether and dried. The residues were weighed, composited and subjected to the furfural test according to the AOAC procedure (4).

The data are shown in graphic form in figure 1. Solution and precipitation of hemicelluloses are usually associated with basic and acidic media, respectively. These data, however, indicate that 5.0 M perchloric acid is positive but not selective in action, that the removal of total solids proceeds at a faster rate than the removal of xylan, that the maximum loss of total solids is only about 80 % of the xylan present, that the xylan removed constitutes approximately 68 % of the total loss, and finally that after two hours of contact less than 60 % of the xylan has been removed. A 2.5 M solution was less effective as a solvent but followed a similar pattern. Complete extraction of xylan by 5.0 M acid would require several hours at best. In the meantime it is almost certain that considerable hydrolysis would have occurred. It is believed, however, that holocellulose affords a highly suitable substrate for the solubility tests inasmuch as the preliminary treatments removed some encrusting substances. Nevertheless, the data indicate that the cellulosans are firmly bound and that this removal is accompanied by loss of hexosan. The data presented here and elsewhere (5) indicate that a satisfactory removal of furfural-yielding substances from most celluloses is perhaps unattainable and really undesirable by chemical means. The problem is really to fractionate on the basis of chain lengths rather than on a qualitative basis.

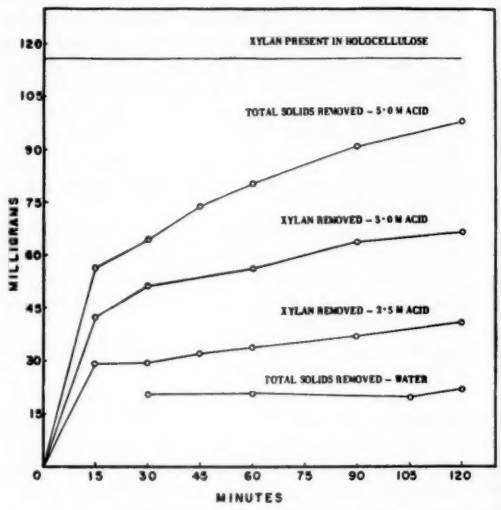

FIG. 1. The weight of total solids and xylan removed from cornstalk holocellulose by aqueous perchloric acid at 25° C for periods up to two hours.

SUMMARY

Hemicelluloses are soluble in aqueous perchloric acid. The total solids removed by 5.0 M perchloric acid were about two-thirds xylan and one-third hexosan; about 60 % of the xylan was removed, thus indicating the positive but nonselective and incomplete solvent action of 5.0 M perchloric acid.

LITERATURE CITED

1. NIELSEN, J. P. and GLEASON, P. C. Rapid determination of starch. Ind. Eng. Chem., Anal. Ed. 17: 131–134. 1945.
2. PUCHER, G. W., LEAVENWORTH, C. S., VICKERY, H. B. Determination of starch in plant tissues. Anal. Chem. 20: 850–853. 1948.
3. BENNETT, EMMETT. Preparation of holocellulose from nonwoody plant material. Anal. Chem. 19: 215. 1947.
4. ASSOCIATION OF OFFICIAL AGRICULTURAL CHEMISTS Official and Tentative Methods of Analysis, 6th Ed. P. 412. Assoc. Offic. Agr. Chemists, Washington 4, D. C. 1945.
5. NORMAN, A. G. The association of xylan with cellulose in certain structural celluloses. Biochem. Jour. 30: 2054–2072. 1936.

[1] Received September 27, 1955.
[2] Contribution No. 1009, University of Massachusetts Agricultural Experiment Station, Amherst, Massachusetts.

GENERAL INDEX

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